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PHYSICAL STUDIES OF CHOLESTEROL-PHOSPHOLIPID AND CHOLESTEROL-GLYCOLIPID INTERACTIONS IN MODEL AND *ACHOLEPLASMA LAIDLAWII* B MEMBRANES

BY

TODD PATRICK WILLIAM MCMULLEN

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Ronald N. McElhaney
Dr. Ronald N. McElhaney (Supervisor)

D.N. Brindley
Dr. David N. Brindley (Chairperson)

Robert O. Ryan
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It is not from space that I must seek my dignity, but from the government of my thought. I shall have no more if I possess worlds. By space the universe encompasses and swallows me up like an atom; by thought I comprehend the world.

Blaise Pascal, *Pensées*

To my parents Gail and Gene.

To my sisters Tracy and Sherri.

ABSTRACT

Cholesterol, as a major structural component of the plasma membranes of eukaryotic cells, plays a crucial role in modulating the physical properties of the host membrane bilayer. Using a combination of differential scanning calorimetry, Fourier transform infrared and ^{31}P-nuclear magnetic resonance spectroscopy, I investigated the nature of cholesterol-phospholipid and cholesterol-glycolipid interactions in binary model membrane systems by systematically altering the structure of the lipid polar headgroup and the length and saturation of the lipid hydrocarbon chains, as well as the length of the cholesterol alkyl side chain. This investigation revealed that the stoichiometry and nature of cholesterol-lipid interactions vary significantly with changes to the polar or non-polar regions of the host phospholipid bilayer. Specifically, cholesterol-lipid interactions are favored with minimal mismatch between the hydrophobic lengths of the cholesterol and lipid molecules, and when the lipid polar headgroups do not exhibit extensive hydrogen bonding or electrostatic interactions. Consequently, cholesterol is fully miscible in the host bilayer and cholesterol exerts a profound effect on the physical properties of the host bilayer membrane. Conversely, the miscibility of cholesterol, and the subsequent effect of cholesterol on the physical properties of the host bilayer, is markedly reduced in lipid bilayers where the degree of cholesterol-lipid hydrophobic mismatch is large and the lipid polar headgroups exhibit extensive inter-headgroup hydrogen bonding and electrostatic interactions. Thus prior models of cholesterol-lipid interactions in biological membranes, based almost exclusively on studies of cholesterol/phosphatidylcholine mixtures, are inadequate and I proposed a new model for cholesterol-lipid interactions to account for

cholesterol-lipid hydrophobic mismatch and lipid headgroup interactions. I tested this model for cholesterol-lipid interactions in a biological system, *Acholeplasma laidlawii* B, and revealed that differences in the affinity of cholesterol for different phospholipids or glycolipids can result in the formation of distinct cholesterol-rich and cholesterol-poor lipid domains in *A. laidlawii* B membranes. My work, coupled with the recent discovery of a number of membrane proteins which interact specifically with cholesterol-rich membranes, suggests that cholesterol-rich and cholesterol-poor domains play a critical role in the structure and function of biological membranes.

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TABLE OF CONTENTS

Page

CHAPTER I. GENERAL INTRODUCTION 1
- The biology of sterols 1
  - The structure and function of sterols in eukaryotic cells 1
  - Biosynthesis of cholesterol 2
- The biology of membranes 4
  - The function of biomembranes 4
  - The composition of biomembranes 4
  - The structure of biomembranes 5
- The effect of cholesterol on biomembrane structure and function 8
  - Function of cholesterol in biomembranes 8
  - Cholesterol-phospholipid interactions 10
- THESIS OBJECTIVE 12
- Experimental methodology 12
- Lipid phase behavior and model bilayer membranes 13
  - Techniques for the study of the thermotropic phase behavior and organization of model and *A. laidlawii* B membranes 15
    - Differential scanning calorimetry 15
    - Fourier transform infrared spectroscopy 17
    - ^{31}P-Nuclear magnetic resonance spectroscopy 18
- REFERENCES 28

CHAPTER II. DIFFERENTIAL SCANNING CALORIMETRIC STUDY OF THE EFFECT OF CHOLESTEROL ON THE THERMOTROPIC PHASE BEHAVIOR OF A HOMOLOGOUS SERIES OF LINEAR SATURATED PHOSPHATIDYLCHOLINES 40
- INTRODUCTION 40
- MATERIALS AND METHODS 43
- RESULTS 44

Page

Thermotropic phase behavior of pure phosphatidylcholines 44

The effect of cholesterol on the pretransition 45

The effect of cholesterol on the main phase transition 45

DISCUSSION 47

REFERENCES 70

CHAPTER III. NEW ASPECTS OF THE INTERACTION OF CHOLESTEROL WITH DIPALMITOYLPHOSPHATIDYLCHOLINE BILAYERS AS REVEALED BY HIGH-SENSITIVITY DIFFERENTIAL SCANNING CALORIMETRY 74

INTRODUCTION 74

MATERIALS AND METHODS 77

RESULTS 78

DISCUSSION 82

REFERENCES ...104

CHAPTER IV. COMPARATIVE DIFFERENTIAL SCANNING CALORIMETRIC AND FTIR AND ^{31}P-NMR SPECTROSCOPIC STUDIES OF THE EFFECT OF CHOLESTEROL AND ANDROSTENOL ON THE THERMOTROPIC PHASE BEHAVIOR AND ORGANIZATION OF PHOSPHATIDYLCHOLINE BILAYERS ...109

INTRODUCTION ...109

MATERIALS AND METHODS ...112

RESULTS ...114

Calorimetric studies of the thermotropic phase behavior of the pure phosphatidylcholines ...114

Effect of androstenol and cholesterol on the pretransition ...115

Effect of androstenol and cholesterol on the main transition ...115

FTIR studies of androstenol/PC and cholesterol/PC mixtures ...117

^{31}P-NMR studies of androstenol/PC and cholesterol/PC mixtures ...121

Page
DISCUSSION ...123
REFERENCES ...150

CHAPTER V. DIFFERENTIAL SCANNING CALORIMETRIC STUDY OF THE EFFECT OF STEROL SIDE CHAIN LENGTH AND STRUCTURE ON DIPALMITOYLPHOSPHATIDYLCHOLINE THERMOTROPIC PHASE BEHAVIOR ...154
INTRODUCTION ...154
MATERIALS AND METHODS ...158
RESULTS ...159
DISCUSSION ...161
REFERENCES ...182

CHAPTER VI. THE EFFECT OF SIDE CHAIN ANALOGUES OF CHOLESTEROL ON THE THERMOTROPIC PHASE BEHAVIOR OF 1-STEAROYL-2-OLEOYL-PHOSPHATIDYLCHOLINE BILAYERS: A DIFFERENTIAL SCANNING CALORIMETRIC STUDY ...186
INTRODUCTION ...186
MATERIALS AND METHODS ...188
RESULTS ...189
DISCUSSION ...192
REFERENCES ...216

CHAPTER VII. CALORIMETRIC AND SPECTROSCOPIC STUDIES OF THE EFFECTS OF CHOLESTEROL ON THE THERMOTROPIC PHASE BEHAVIOR AND ORGANIZATION OF A HOMOLOGOUS SERIES OF LINEAR SATURATED PHOSPHATIDYLETHANOLAMINE BILAYERS ...220
INTRODUCTION ...220
MATERIALS AND METHODS ...223

Page

RESULTS ...226

Thermotropic phase behavior of pure phosphatidylethanolamines ...226

Effect of cholesterol on the thermotropic phase behavior and organization of DMPE, DPPE and DSPE bilayers: Calorimetric studies ...227

Effect of cholesterol on the thermotropic phase behavior and organization of DMPE, DPPE and DSPE bilayers: FTIR spectroscopic studies ...230

Effect of cholesterol on the thermotropic phase behavior and organization of DMPE, DPPE and DSPE bilayers: ^{31}P-NMR spectroscopic studies ...234

DISCUSSION ...235

REFERENCES ...266

CHAPTER VIII. CALORIMETRIC AND SPECTROSCOPIC STUDIES OF THE EFFECTS OF CHOLESTEROL ON THE THERMOTROPIC PHASE BEHAVIOR AND ORGANIZATION OF A HOMOLOGOUS SERIES OF LINEAR SATURATED PHOSPHATIDYLSERINE BILAYERS ...272

INTRODUCTION ...272

MATERIALS AND METHODS ...275

RESULTS ...277

Effect of cholesterol on the thermotropic phase behavior and organization of DMPS, DPPS and DSPS bilayers: Calorimetric studies ...277

Effect of cholesterol on the thermotropic phase behavior and organization of DMPS, DPPS and DSPS bilayers: FTIR spectroscopic studies ...280

DISCUSSION ...284

REFERENCES ...317

Page

CHAPTER IX. DIFFERENTIAL SCANNING CALORIMETRIC STUDIES OF THE INTERACTION OF CHOLESTEROL WITH DISTEAROYL- AND DIELAIDOYL-PHOSPHATIDYLCHOLINE, PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLSERINE ...323

INTRODUCTION ...323

MATERIALS AND METHODS ...325

RESULTS ...327

Thermotropic phase behavior of cholesterol/phosphatidylcholine mixtures ...327

Thermotropic phase behavior of cholesterol/phosphatidylethanolamine mixtures ...328

Thermotropic phase behavior of cholesterol/phosphatidylserine mixtures ...329

DISCUSSION ...331

REFERENCES ...356

CHAPTER X. DIFFERENTIAL SCANNING CALORIMETRIC STUDY OF THE INTERACTION OF CHOLESTEROL WITH THE MAJOR LIPIDS OF THE *ACHOLEPLASMA LAIDLAWII* B MEMBRANE ...361

INTRODUCTION ...361

MATERIALS AND METHODS ...364

RESULTS ...365

DISCUSSION ...371

REFERENCES ...403

Page

CHAPTER XI. GENERAL DISCUSSION AND CONCLUSIONS ...408
Cholesterol-phospholipid interactions in model membrane systems ...409
Cholesterol-phospholipid interactions in biological membranes ...412
Cholesterol-protein interactions in biological membranes ...415
The direction of future investigations ...417
REFERENCES ...421

LIST OF TABLES

Page

Table I-1. Lipid compositions of subcellar organelles from rat liver. Values in weight% except for cholesterol, where values are presented as a molar fraction of the total phospholipids. 26

Table I-2. Transbilayer lipid asymmetry in human erythrocyte and rat liver plasma membranes. Values in weight %. 27

Table IV-1. Chain length-dependence of the androstenol/CnPC anomalous endotherm transition temperature and size. ...149

Table X-1. The polar headgroup composition of the membrane lipids from *A. laidlawii* cells grown in the presence of exogenous elaidic acid and avidin. ...402

LIST OF FIGURES

Page

Figure I-1. Full generalized structure of the dominant sterols of most eukaryotic organisms. 21

Figure I-2. Structure of the cholesterol molecule, the dominant sterol in higher eukaryotes. 23

Figure I-3. Representative structures for selected phospholipid molecules. 25

Figure II-1. Representative DSC scans of unannealed DPPC bilayers containing various levels of cholesterol 53

Figure II-2. Optimized scans of the pretransition phase transition of DPPC vesicles containing various levels of cholesterol. 55

Figure II-3. Sample deconvolution of the DSC endotherm for three concentrations of cholesterol in DPPC vesicles. 57

Figure II-4. Representative plot of the net decrease in the temperature of the sharp component of the DSC endotherm as a function of cholesterol concentration. 59

Figure II-5. Representative plots of the enthalpy of the sharp component of the DSC endotherm as a function of cholesterol concentration for the PC homologous series. 61

Page

Figure II-6. Representative plots of the enthalpy of the broad component of the DSC endotherm as a function of cholesterol concentration for the PC homologous series. 63

Figure II-7. Representative plots of the overall main phase transition enthalpies of PC's as a function of increasing cholesterol concentration. 65

Figure II-8. The net transition temperature shift for each PC broad component is shown as a function of increasing cholesterol concentration. 67

Figure II-9. Representative plots of the $\Delta T_{1/2}$ of the broad component of the main phase transition as a function of increasing cholesterol levels. 69

Figure III-1. The cholesterol/DPPC phase diagram proposed by Vist and Davis (1990). 89

Figure III-2. Raw representative DSC scans of annealed DPPC bilayers of various cholesterol molar concentrations. 91

Figure III-3. Representative decomposed endotherms of the main phase transition of DPPC with various amount of cholesterol. 93

Figure III-4. Plots of the temperature, enthalpy and the cooperativity of the overall, as well as broad and sharp components, of the main chain-melting transition of cholesterol/DPPC mixtures. 95

Figure III-5. Representative plots of the endotherm subtraction of pure DPPC from cholesterol-containing DPPC bilayers. 97

Page

Figure III-6. Overall and decomposed plots of DMPC, DSPC and DAPC endotherms with various levels of cholesterol. ...99

Figure III-7. Overall $\Delta T_{1/2}$'s of DMPC, DPPC, DSPC and DAPC plotted as a function of increasing cholesterol levels. ...101

Figure III-8. Temperature/composition diagram for cholesterol/DPPC mixtures as a function of increasing temperature and cholesterol levels. ...103

Figure IV-1. Representative DSC scans of unannealed DMPC bilayers with various concentrations of cholesterol or androstenol. ...128

Figure IV-2. Representative DSC scans of unannealed DPPC bilayers with various concentrations of cholesterol or androstenol. ...130

Figure IV-3. Representative DSC scans of unannealed DSPC bilayers with various concentrations of cholesterol or androstenol. ...132

Figure IV-4. A comparison of the DPPC and DSPC main transition enthalpies as a function of increasing sterol concentrations. ...134

Figure IV-5. Dependence of the $\Delta T_{1/2}$'s of the broad component of sterol/DPPC and sterol/DSPC mixtures on increasing sterol concentrations. ...136

Figure IV-6. Plot of the broad component transition temperature shift as a function of increasing sterol concentrations for short, intermediate and long chain PCs. ...138

Page

Figure IV-7. A comparison of FTIR and DSC data for pure DMPC and selected androstenol/DMPC and cholesterol/DMPC mixtures. ...140

Figure IV-8. A comparison of FTIR and DSC data for pure DSPC and selected androstenol/DSPC and cholesterol/DSPC mixtures. ...142

Figure IV-9. A comparison of the CD_2 asymmetric stretching absorption maxima as a function of temperature for bilayers composed of 14:0 PC-d_{54} and 14:0 PC-d_{54} with 25 mol% androstenol or 25 mol% cholesterol. ...144

Figure IV-10. Comparative stacked plots of ^{31}P-NMR spectra for DMPC pure and DMPC with 25 mol% androstenol or 25 mol% cholesterol. ...146

Figure IV-11. Comparative stacked plots of ^{31}P-NMR spectra for DSPC pure and DSPC with 35 mol% androstenol or 35 mol% cholesterol. ...148

Figure V-1. Sterol side chain structures and their abbreviations. ...167

Figures V-2a,b. Representative plots of DPPC bilayer endotherms as a function of increasing C-22 (A) and *i*-C10 (B) sterol concentration. ...169

Figures V-3a. Three dimensional plot of the effect of increasing sterol concentrations on the DPPC main transition overall enthalpy for the *n*-series. ...171

Figures V-3b. Three dimensional plot of the effect of increasing sterol concentrations on the DPPC main transition overall enthalpy for the *iso*-series. ...172

Page

Figures V-4a. Sharp component enthalpy as a function of increasing sterol concentration for the *n*-series. ...174

Figures V-4b. Sharp component enthalpy as a function of increasing sterol concentration for *iso*-series. ...175

Figures V-5a,b. Sharp component transition temperature as a function of increasing sterol concentration for the *n*- (A) and *iso*-series (B). ...177

Figures V-6a,b. Broad component transition temperature as a function of increasing sterol concentration for the the *n*- (A) and *iso*-series (B). ...179

Figures V-7a,b. Overall DPPC endotherm $\Delta T_{1/2}$ as a function of increasing sterol concentrations for the the *n*- (A) and *iso*-series (B). ...181

Figure VI-1. Sterol side chain structures and their abbreviations. ...197

Figures VI-2a,b. Representative DSC thermograms of SOPC bilayers containing increasing amounts of (**A**) C-22 and (**B**) *i*-C10 sterols. ...199

Figures VI-3a. Three dimensional plot of the effect of increasing sterol concentration on the overall enthalpy of the SOPC main transition for the *n*-series. ...201

Figures VI-3b. Three dimensional plot of the effect of increasing sterol concentration on the overall enthalpy of the SOPC main transition for the *iso*-series. ...202

Page

Figures VI-4a. Sharp component enthalpy of sterol/SOPC mixtures as a function of increasing sterol concentration for the *n*-series. ...204

Figures VI-4b. Sharp component enthalpy of sterol/SOPC mixtures as a function of increasing sterol concentration for the *iso*-series. ...205

Figures VI-5a,b. Sharp component transition temperature of sterol/SOPC mixtures as a function of increasing sterol concentration for the (**A**) *n*- and (**B**) *iso*-series. ...207

Figures VI-6a,b. Broad component transition temperature of sterol/SOPC mixtures as a function of increasing sterol concentration for the (**A**) *n*- and (**B**) *iso*-series. ...209

Figures VI-7a,b. Overall $\Delta T_{1/2}$ of sterol/SOPC endotherms as a function of increasing sterol concentration for the (**A**) *n*- and (**B**) *iso*-series. ...211

Figure VI-8. Broad component transition temperature shifts for both SOPC and DPPC bilayers containing cholesterol, C-22 and *i*-C10 sterols as a function of increasing sterol concentration. ...213

Figure VI-9. Plots of the % remaining enthalpy of the overall chain-melting transition for SOPC and DPPC bilayers as a function of increasing C-22 sterol levels. ...215

Figure VII-1. Representative heating and cooling thermograms of DMPE bilayers containing progressively increasing levels of cholesterol. ...243

Page

Figure VII-2. Representative heating and cooling thermograms of DPPE bilayers containing progressively increasing levels of cholesterol. ...245

Figure VII-3. Representative heating and cooling endotherms of DSPE bilayers containing progressively increasing levels of cholesterol. ...247

Figure VII-4. Plots of the temperature of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DMPE, cholesterol/DPPE and cholesterol/DSPE mixtures. ...249

Figure VII-5. Plots of the overall enthalpy of the chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DMPE, cholesterol/DPPE and cholesterol/DSPE mixtures. ...251

Figure VII-6. Representative FTIR CH_2 stretching, C=O stretching and CH_2 bending absorption bands for the L_α, L_β, L_c1 and L_c2 phases of fully hydrated *n*-saturated diacyl PE bilayers. ...253

Figure VII-7. Representative FTIR C=O stretching and CH_2 bending absorption bands for DMPE bilayers containing >30 mol% cholesterol and DMPE bilayers containing >10 mol% cholesterol. ...255

Figure VII-8. Representative FTIR C=O stretching and CH_2 bending absorption bands for DSPE bilayers containing various levels of cholesterol. ...257

Figure VII-9. A comparative plot of the HS-DSC thermograms and the corresponding absorption maxima of the CH_2 symmetric stretching bands of DMPE, DPPE and DSPE bilayers containing 35 mol% cholesterol. ...259

Page

Figure VII-10. Representative ^{31}P-NMR spectra of pure PE bilayer L_α, L_β and L_c phases. ...261

Figure VII-11. Representative ^{31}P-NMR spectra of DMPE bilayers containing >30 mol% cholesterol and DPPE bilayers containing >10 mol% cholesterol. ...263

Figure VII-12. Representative ^{31}P-NMR spectra of DSPE/cholesterol mixtures. ...265

Figure VIII-1. Representative heating and cooling thermograms of DMPS bilayers containing progressively increasing levels of cholesterol. ...290

Figure VIII-2. Plot of the transition temperature of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DMPS mixtures. ...292

Figure VIII-3. Plot of the enthalpy of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DMPS mixtures...294

Figure VIII-4. Representative heating and cooling thermograms of DPPS bilayers containing progressively increasing levels of cholesterol. ...296

Figure VIII-5. Plots of the transition temperature of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol-DPPS mixtures. ...298

Figure VIII-6. Plots of the enthalpy of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DPPS mixtures. ...300

Page

Figure VIII-7. Representative heating and cooling endotherms of DSPS bilayers containing progressively increasing levels of cholesterol. ...302

Figure VIII-8. Plots of the transition temperature of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol-DSPS mixtures. ...304

Figure VIII-9. Plots of the enthalpy of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DMPS mixtures...306

Figure VIII-10. Representative FTIR CH_2 stretching, C=O stretching and CH_2 bending absorption bands for the L_α, L_β, L_c1 and L_c2 phases of fully hydrated *n*-saturated diacyl PS bilayers. ...308

Figure VIII-11. Representative FTIR C=O stretching and CH_2 bending absorption bands for DMPS bilayers containing cholesterol. ...310

Figure VIII-12. Representative FTIR C=O stretching and CH_2 bending absorption bands for DMPS, DPPS and DSPS bilayers containing cholesterol. ...312

Figure VIII-13. Representative FTIR C=O stretching and CH_2 bending absorption bands for DPPS bilayers containing cholesterol and DSPS bilayers containing from 0 to 25 mol% cholesterol. ...314

Figure VIII-14. Representative FTIR C=O stretching and CH_2 bending absorption bands for DSPS bilayers containing cholesterol levels exceeding 35 mol%....316

Page

Figure IX-1. Representative DSC heating scans of aqueous dispersions of DSPC and DEPC bilayers containing various concentrations of cholesterol. ...335

Figure IX-2. Representative plots of the temperatures of the DSPC and DEPC chain-melting transitions. ...337

Figure IX-3. Representative plots of the total enthalpy for the DSPC and DEPC chain-melting transitions as a function of increasing cholesterol levels. ...339

Figure IX-4. Representative DSC heating and cooling scans of aqueous dispersions of the DSPE bilayers containing various concentrations of cholesterol. ...341

Figure IX-5. Representative DSC heating and cooling scans of aqueous dispersions of the DEPE bilayers containing various concentrations of cholesterol. ...343

Figure IX-6. Representative plots of the temperatures of the phase transitions of DSPE and DEPE bilayers as a function of increasing cholesterol levels. ...345

Figure IX-7. Representative plots of the enthalpy of the phase transitions of DSPE and DEPE bilayers as a function of increasing cholesterol levels. ...347

Figure IX-8. Representative DSC heating and cooling scans of aqueous dispersions of the DSPS bilayers containing various concentrations of cholesterol. ...349

Figure IX-9. Representative DSC heating and cooling scans of aqueous dispersions of the DEPS bilayers containing various concentrations of cholesterol. ...351

Figure IX-10. Representative plots of the temperatures of the phase transitions of DSPS and DEPS bilayers as a function of increasing cholesterol levels. ...353

Page

Figure IX-11. Representative plots of the total enthalpy of the phase transitions of DSPS and DEPS bilayers as a function of increasing cholesterol levels. ...355

Figure X-1. Representative DSC heating and cooling scans of aqueous dispersions of the PG from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol. ...379

Figure X-2. Representative plots of the temperatures of the broad and sharp components of the main phase transition of aqueous dispersions of the PG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of increasing cholesterol levels. ...381

Figure X-3. Representative plots of the total enthalpy and the enthalpy of the sharp and broad components of the main phase transition of aqueous dispersions of the PG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. ...383

Figure X-4. Representative DSC heating and cooling scans of aqueous dispersions of the DGDG from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol. ...385

Figure X-5. Representative plots of the temperatures of the phase transitions of aqueous dispersions of DGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. ...387

Figure X-6. Representative plots of the total enthalpy of the phase transitions of aqueous dispersions of the DGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. ...389

Page

Figure X-7. Representative DSC heating and cooling scans of aqueous dispersions of the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol. ...391

Figure X-8. Representative plots of the temperatures of the phase transitions of aqueous dispersions of the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. ...393

Figure X-9. Representative plots of the total enthalpy of the phase transitions of aqueous dispersions of the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. ...395

Figure X-10. Representative DSC heating scans of aqueous dispersions of the total polar lipids from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol. ...397

Figure X-11. Representative plot of the overall chain-melting phase transition midpoint temperature of aqueous dispersions of the total polar lipids from elaidic acid-homogeneous *A. laidlawii* membranes as a function of increasing cholesterol levels. ...399

Figure X-12. Representative plot of the total enthalpy of the chain-melting phase transition of aqueous dispersions of the total polar lipids from elaidic acid-homogeneous *A. laidlawii* membranes as a function of increasing cholesterol levels. ...401

Figure XI-1. The lateral organization of cholesterol in different phospholipid bilayers. ...420

LIST OF ABBREVIATIONS

GENERAL

ACAT	acyl-coenzyme A:cholesterol acyltransferase
CEH	acyl cholesterol ester hydrolase
DNA	deoxyribonucleic acid
HDL	high-density lipoprotein
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
LDL	low-density lipoprotein
SCAP	SREBP cleavage-activating protein
SBP	sterol-binding protein
SREBP	sterol regulatory element binding proteins (1 and 2)
TLC	thin-layer chromatography

LIPIDS

APG	acyl polyprenylglucoside
DAPC	diarachidoylphosphatidylcholine (20:0 PC)
DEPC	dielaidoylphosphatidylcholine ($18:1_t$ PC)
DEPE	dielaidoylphosphatidylethanolamine ($18:1_t$ PE)
DEPS	dielaidoylphosphatidylserine ($18:1_t$ PS)
DGDG	diglucosyldiacylglycerol
DLPC	dilaurylphosphatidylcholine (12:0 PC)
DMPC	dimyristoylphosphatidylcholine (14:0 PC)
DMPE	dimyristoylphosphatidylethanolamine (14:0 PE)
DMPS	dimyristoylphosphatidylserine (14:0 PS)

DOPC	dioleoylphosphatidylcholine ($18:1_c$ PC)
DOPE	dioleoylphosphatidylethanolamine ($18:1_c$ PE)
DPPC	dipalmitoylphosphatidylcholine (16:0 PC)
DPPE	dipalmitoylphosphatidylethanolamine (16:0 PE)
DPPS	dipalmitoylphosphatidylserine (16:0 PS)
DSPC	distearoylphosphatidylcholine (18:0 PC)
DSPE	distearoylphosphatidylethanolamine (18:0 PE)
DSPS	distearoylphosphatidylserine (18:0 PS)
EYPE	egg yolk phosphatidylethanolamine
GalCer	galactoceramide
GPDGDG	glyceryl-phosphoryldiglucosyldiacylglycerol
GPMGDG	glycerylphosphorylmonoglucosyldiacylglycerol
MGDG	monoglucosyldiacylglycerol
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PS	phosphatidylserine
POPE	1-palmitoyl-2-oleoyl-phosphatidylethanolamine ($16:0/18:1_c$ PE)
POPS	1-palmitoyl-2-oleoyl-phosphatidylserine ($16:0/18:1_c$ PS)
n(iso)-C#	refers to C17 sterol side chain where *n*- specifies unbranched and *iso*-specifies terminal methyl branch, and # specifies number of carbon atoms
(n)PC	specific phosholipids are designated by the notation n:0 PC, where n is the number of carbon atoms per hydrocarbon chain with the zero indicating the absence of double bonds. PC represents phosphatidylcholine

SOPC	1-steroyl-2-oleoyl-phosphatidylcholine ($18{:}0/18{:}1_c$ PC)
SOPS	1-steroyl-2-oleoyl-phosphatidylserine ($18{:}0/18{:}1_c$ PS)
SPM	sphingomyelin

LIPID PHASES

L_α	lamellar liquid-crystalline phase
L_β	lamellar gel phase (when ′ present, tilted hydrocarbon chains)
L_c	lamellar crystalline phase; type 1, stable phase, type 2, metastable crystalline phase (when ′ present, tilted hydrocarbon chains).
$L_{o\alpha}$	cholesterol/PC liquid-ordered liquid crystalline-like phase
$L_{o\beta}$	cholesterol/PC liquid-ordered gel-like phase
P_{β}'	rippled gel phase with tilted hydrocarbon chains
H_{II}	inverted hexagonal phase.

TECHNIQUES

^{13}C-NMR	carbon-13 nuclear magnetic resonance
^{2}H-NMR	deuterium nuclear magnetic resonance spectroscopy
^{31}P-NMR	phosphorus-31 nuclear magnetic resonance
ESR	electron spin resonance
FTIR	Fourier transform infrared
LS/HS-DSC	low-sensitivity or high-sensitivity differential scanning calorimetry
PPM	parts per million
ΔH	transition enthalpy

$\Delta T_{1/2}$	the width of the phase transition at half-height, inversely related to cooperativity by the equation $\Delta H_{vh}=4RT_m^2/\Delta T_{1/2}$
T_m	the phase transition temperature (measured as the temperature of the peak in the DSC endotherm)

CHAPTER I. GENERAL INTRODUCTION

The Biology of Sterols

The structure and function of sterols in eukaryotic cells

Sterols are cyclic, polyterpenoid-derived, weakly amphiphilic molecules found only in eukaryotic organisms (Nes, 1973; Bloch, 1983). Sterols exhibit considerable structural diversity, presumably due to the unique demands placed upon sterols in different organisms in different environments (Nes, 1973). However, the vast majority of sterols share a common structural theme, a planar cyclopentano-perhydrophenanthrecene ring system with a β-hydroxy group at carbon 3 and an alkyl side chain at carbon 17, as shown in Figure I-1 (Nes, 1973). The dominant sterol in higher animals, cholesterol, is shown in Figure I-2 (Nes and McKean, 1977). Cholesterol is a major component of the plasma membranes of animal cells and also occurs in smaller quantities in the membranes of some subcellular organelles. Free cholesterol is also found in high quantities in the surface monolayers of the blood plasma lipoproteins and fatty acid esters of cholesterol are major constituents of the hydrophobic cores of such particles (Faust *et al.*, 1988). In addition to its structural role in membranes and in blood lipoprotein particles, cholesterol is also the biosynthetic precursor to the steroid hormones which regulate a wide variety of important physiological functions, and to the essential fat-soluble vitamin D (Stryer, 1988). As well, cholesterol degradation results in the formation of the bile acids, detergent-like compounds which perform an important role in lipid digestion and absorption. Although cholesterol has been maligned due to its role in the development of heart disease in humans, it is important to remember that this sterol is required for the growth of the cells of all higher animals and that a block in its biosynthetic pathway is fatal unless exogenous cholesterol is supplied. Finally, the important role of cholesterol in human physiology is perhaps best illustrated by the fact that 13 Nobel prizes have been awarded for insights into the structure, biosynthesis and function of this small molecule (Brown and Goldstein, 1986).

Cholesterol has a multifunctional role in eukaryotic cells. Cholesterol modulates the phase state, fluidity and physical properties of the membranes of eukaryotic cells. In addition, cholesterol may also regulate the activity of membrane proteins via bulk changes in the physical properties of the membrane or via specific cholesterol-protein interactions (Nes, 1973; Dahl and Dahl, 1988; Yeagle, 1988; Parks *et al.*, 1995). Furthermore, there is a close association between the availability or synthesis of cholesterol and the processes of cell metabolism and growth (Bloch, 1983; Dahl and Dahl, 1988). Cholesterol synthesis precedes phospholipid and DNA[1] synthesis in growing cells and there is evidence that the non-sterol products of the isoprenoid pathway, and perhaps cholesterol itself, perform regulatory roles in the processes of mammalian cell metabolism, cycling and proliferation (Brown and Goldstein, 1974; Chen *et al.*, 1974, 1975; Kandutsch and Chen, 1977; Siperstein, 1984; Bloch, 1983; Dahl and Dahl, 1988). Finally, it is important to note that animal cells have a stringent requirement for the structural features of cholesterol, and even closely related cholesterol analogues do not support growth in sterol-auxotrophic fibroblasts (Rujanavech and Silbert, 1986; Dahl and Dahl, 1988).

Biosynthesis of cholesterol

Having addressed the importance of cholesterol in mammalian cell physiology, we now examine how cells obtain cholesterol and regulate cellular cholesterol levels. Under normal conditions, a cell can obtain cholesterol from both endogenous and exogenous sources which operate simultaneously. The endogenous cellular pathway involves the energetically expensive *de novo* synthesis of cholesterol at the endoplasmic reticulum in which the membrane-anchored HMG Co A reductase is the key rate-limiting enzyme (Schroepfer, 1982; Bloch, 1983; Faust *et al.*, 1988; Goldstein and Brown, 1990; Liscum and Dahl, 1992; van Meer, 1993). Exogenous supplies of cholesterol are obtained via lipoproteins circulating in the blood plasma of which LDL particles account for most of the transfer of cholesterol to extrahepatic cells (Goldstein and Brown, 1977; Brown and Goldstein, 1986; Bradley and Gianturco, 1988; Liscum and Dahl, 1992). Cellular levels of cholesterol are tightly controlled by the coordinated regulation of exogenous and endogenous supplies of cholesterol, as well as cholesterol storage and efflux. For example, when cellular levels of cholesterol become excessive, HMG-CoA reductase activity is downregulated via decreased gene expression and increased proteolytic turnover of the enzyme (Chang *et al.*, 1986; Faust *et al.*, 1988; Goldstein and Brown, 1990; Lutton, 1991; Wang *et al.*, 1994; Lange and Steck, 1994, 1996). In addition, the number of LDL receptors in the cell decrease and ACAT, the enzyme responsible for the formation of cholesteryl esters for storage of cholesterol, is upregulated (Brown and Goldstein, 1986; Lutton, 1991; Liscum and Dahl, 1992; Wang *et al.*, 1994; Yang *et al.*, 1994; Chang *et al.*, 1986, 1995; Uelmen *et al.*, 1995; Lange and Steck, 1996). Conversely, when cholesterol levels are insufficient to meet the requirements of the cell, HMG-CoA reductase activity and LDL receptor levels increase and CEH is upregulated to release free cholesterol from cholesteryl ester inclusion vesicles. The level of cholesterol within the cell can also be modulated by reverse cholesterol transport via spontaneous transfer of cholesterol from the plasma membrane to HDL (Glomset, 1968; Karlin *et al.*, 1987; Johnson *et al.*, 1991; Schroeder *et al.*, 1991; Rothblat *et al.*, 1992; Kilsdonk *et al.*, 1992; Davidson, 1995). The homeostatic mechanism(s) within the cell which sense the level of cholesterol within cellular membranes, and then coordinate changes in endogenous metabolism, exogenous uptake/efflux and the storage of cholesterol, are unknown. However a number of different factors have been shown to modulate cellular levels including cholesterol, the closely related oxysterols 25, 26 and 27-hydroxycholesterol, steroid hormones and the cholesterol/lipid ratio of the endoplasmic reticulum (Brown and Goldstein, 1974; Brown and Goldstein, 1986; Davis and Poznansky, 1987; Goldstein and Brown, 1990; Lutton, 1991; Liscum and Dahl, 1992; Liscum and Underwood, 1995; Lange and Steck, 1994, 1996). In addition the endoplasmic reticulum membrane-bound transcription factors SREBP-1 and -2 have been shown to be key elements in the regulation of the activity of HMG CoA reductase and the level of LDL receptors in animal cells (Yokoyama *et al.*, 1993; Yang *et al.*, 1994; Hua *et al.*, 1995). The activities of SREBP-1 and -2 are regulated by a protease, which in turn is sensitive to intracellular membrane sterol levels (Yang *et al.*, 1994). Thus the evidence suggests the existence of a membrane localized mechanism for cholesterol homeostasis in which cholesterol, and possibly the phospholipid composition of the endoplasmic reticulum membrane, may govern the activities of membrane proteins which in turn modulate cholesterol metabolism in the cell (Lange and Steck, 1994, 1996; Wang *et al.*, 1994; Yang *et al.*, 1994; Chang *et al.*, 1995; Uelmen *et al.*, 1995).

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[1] See abbreviations

The Biology of Membranes

The function of biomembranes

Before we can examine the role of cholesterol in biomembranes, we must define the fundamental functions of biomembranes. Functional specialization during the course of evolution is closely linked to the formation of compartments. Biological membranes define cells, and the organelles within a cell, by providing a partially non-permeable lipid boundary to maintain the unique environment in which the contents of the cell, or organelle, optimally function (Singer and Nicholson, 1972). Imbedded in the membrane bilayer matrix are the membrane proteins which regulate the movement of metabolites in and out of the cell, or organelle, and may also participate in numerous enzymatic and bioenergetic processes (Stryer, 1988; Gennis, 1989). Moreover, the lipid and protein components of biomembranes act as sensors of the extracellular environment and signal transduction and lipid second messenger pathways modulate cell growth, metabolism and function (Stryer, 1988; Gennis, 1989; Spiegel *et al.*, 1996).

The composition of biomembranes

The major components of membranes are proteins and lipids, with a small amount of carbohydrates which are usually in the form of glycoproteins or glycolipids (Gennis, 1989). The relative amounts of the lipids and proteins may vary from 20 % (w/w) protein up to 80% depending on the membrane. In higher eukaryotes, the lipids which comprise the bilayer can be grouped under the following headings: the glycerophospholipids, sphingolipids, glycosphingolipids and cholesterol. Representative structures of three prominent phospholipids in eukaryotic cells, which are also the focus of the present volume of work, are given in Figure I-3 (Hauser and Poupart, 1992). The amphipathic nature of the phospholipids (and of all membrane lipids) is clearly apparent in their chemical structure. Phospholipids consist of a non-polar region comprised of two hydrocarbon chains esterified to glycerol backbone carbons and a polar headgroup which is also attached to the glycerol backbone via a phosphate. In addition to differences in the headgroup structure, the hydrocarbon chains of phospholipids vary considerably in length and degree of unsaturation. It is important to note that eukaryotic biomembranes are typically composed of at least 5 or 6 different phospholipid or glycosphingolipid classes and at least several hundred lipid molecular species when variations with respect to both headgroup and hydrocarbon chain composition are considered (White, 1973; van Meer, 1993). The reason for this large number of unique lipid structures remains one of the fundamental questions in membrane biology (White, 1973; Gennis, 1989; van Meer, 1993). However, the diversity of membrane lipid structures suggests that lipids must have functions more complex than simply the maintenance of membrane bilayer structure.

The structure of cholesterol, shown in Figure I-2, is clearly distinct from that of the phospholipids, sphingolipids and glycosphingolipids. Cholesterol consists of a rigid tetracyclic ring system with two β-methyl substituents at carbons 10 and 13, a β-hydroxyl at carbon 3 and lastly an 8 carbon, isobranched alkyl side chain at carbon 17. Thus, the α-face of cholesterol (Figure I-2) presents a smooth van der Waals surface while the methyl substituents of the sterol ring system are features of the β-face. In a lipid bilayer, the β-hydroxyl group is located at the polar/non-polar interface while the ring system resides in the non-polar region of the bilayer with the alkyl side chain extending towards the bilayer center (Nes and McKean, 1977; Yeagle, 1988).

The structure of biomembranes

The fluid mosaic model of eukaryotic biomembranes proposed by Singer and Nicholson (1972) is perhaps the single most influential concept describing the morphology of biomembranes. This model characterized biomembranes as a "two-dimensional orientated solution of integral membrane proteins...in the viscous phospholipid bilayer." Biomembranes are comprised of two lamellar leaflets with the lipid hydrocarbon chains, or sterol ring system, at the non-polar center with the polar moieties of the lipids facing towards the aqueous milieu. Spanning or partially penetrating into the bilayer are the integral or peripherally associated membrane proteins. Both the lipids and proteins exhibit rapid lateral and rotational motion within the bilayer matrix and, by virtue of this motion, are distributed homogeneously within the plane of the bilayer (Singer and Nicholson, 1972).

While the overall concept of biomembranes as described by the fluid mosaic model is useful, this model fails to adequately describe the asymmetrical and dynamic organization of cell membrane bilayers (Devaux, 1991; Thompson *et al.*, 1992; Thompson, 1993; Vaz and Alameida, 1993; Rogers and Glaser, 1993; Zachowski, 1993; Devaux and Zachowski, 1994; Welti and Glaser, 1994; Jacobsen *et al.*, 1995). Asymmetry is an important aspect of membrane morphology and the phospholipid composition of different membranes varies considerably (see Table I-1), as does the transmembrane distribution of phospholipids within a given membrane (Table I-2) (White, 1973; Zachowski, 1993; van Meer, 1993; Devaux and Zachowski, 1994). Differences in the phospholipid composition of different membranes are regulated by local metabolism and by a combination of specific vesicular and protein-mediated transport mechanisms to and from the endoplasmic reticulum, where most phospholipids are synthesized (Gennis, 1989; van Meer, 1993; Moreau and Cassagne, 1994). With respect to transmembrane asymmetry, differences in the rate of phospholipid flip-flop and the activity of PE- and PS-specific ATP-dependent and non-ATP-dependent flippase proteins are believed to maintain the asymmetric transbilayer distribution of phospholipids in various cellular membranes (Zachowski, 1993; Devaux and Zachowski, 1994). There is also evidence for lateral heterogeneity in biological membranes with membrane phospholipids, or proteins, enriched in particular domains within the plane of the bilayer (Gennis, 1989; Glaser, 1993; Thompson, 1993; Welti and Glaser, 1994; Jacobsen *et al.*, 1995). The mechanisms behind macroscopic domain formation are relatively well documented and rely on the cytoskeleton or specialized protein assemblies such as in polarized epithelial cells (Gennis, 1989; Jacobsen, 1995). Microscopic lateral domains enriched in particular phospholipids or proteins have also been suggested, based on specific phospholipid-protein interactions (Cruetz, 1993; Welti and Glaser, 1994; Jacobsen *et al.*, 1995). The formation of lateral domains in biomembranes could also be a result of mutually insoluble phospholipid or glycosphingolipid phases (Thompson, 1993; Vaz and Alameida, 1993; Welti and Glaser, 1994; Hwang *et al.*, 1995). However, experimental evidence supporting the existence of microscopic phospholipid domains in biological membranes, based solely on the physical chemistry of phospholipids in model membranes, is limited (Vaz and Alameida, 1993; Welti and Glaser, 1994; Hwang *et al.*, 1995). Overall, the asymmetric distribution of phospholipids between different membranes, or within a given membrane, may modulate the function of particular membrane proteins (Devaux and Zachowski, 1994; Welti and Glaser, 1994; Murata *et al.*, 1995; Mosior *et al.*, 1995), lipid second messenger pathways (Speigel *et al.*, 1996), cellular recognition (Devaux and Zachowski, 1994), or the fusogenic potential of a membrane (Gruner *et al.*, 1985; Lindblom and Rilfors, 1989; Devaux, 1991; Gruner, 1992; van Meer, 1993; Welti and Glaser, 1994; Devaux and Zachowski, 1994; Kinnunen *et al.*, 1994). Compositionally distinct lateral domains in biomembranes may also influence bilayer morphology, the interfacial binding and activity of peripheral membrane proteins as well as the efficiency of bimolecular reactions in membranes (Creutz, 1992; Vaz and Alameida, 1993; Thompson, 1993; Burack and Biltonen, 1994; Sigal *et al.*, 1994).

The distribution of cholesterol within eukaryotic intracellular membranes also appears to be asymmetrical (see Table I-1). For example, the endoplasmic reticulum, the site of cholesterol synthesis, contains 10 mol% or less of the total cholesterol found in the membranes of the cell while the plasma membrane typically contains 40 to 50 mol% cholesterol, which is the large majority of the cellular free cholesterol (Yeagle, 1988; Liscum and Dahl, 1992; van Meer, 1993). Considering the relatively fast exchange of cholesterol between membranes compared to other lipids as well as the substantial rate of intermembrane vesicular traffic, maintaining an asymmetric distribution of cholesterol between different membranes is no small task (Bittman, 1988, 1993; van Meer, 1993). Although three different mechanisms for the intracellular transport of cholesterol have been proposed: spontaneous exchange between membranes; vesicular or shuttle transport; or a sterol-binding protein (SBP-2) shuttle system, it is unclear whether one mechanism dominates, or whether these mechanisms work together simultaneously (Liscum and Dahl, 1992; van Meer, 1993; Hapala *et al.*, 1994; Woodford *et al.*, 1995; Liscum and Underwood, 1995; Lange and Steck, 1996). It is also unclear as to whether the transmembrane distribution of cholesterol is symmetrical or asymmetrical, as there is evidence for both cases (Yeagle, 1985, 1988). In addition, a number of investigators have attempted to determine whether cholesterol is distributed homogeneously within the lateral plane of the bilayer, or is distributed among cholesterol-rich and poor phospholipid domains of varying size and composition (Hui, 1988; Schroeder *et al.*, 1991, 1995; Yandouzi and le Grimellec, 1992; Chong, 1994; Mattjus *et al.*, 1994; Tang *et al.*, 1995; Slotte, 1995). The results of these studies are varied and thus the spatial distribution of cholesterol within model and biological membranes remains an open question. However, it is increasingly clear that the distribution of cholesterol within cellular membranes depends on the lipid composition of the host bilayer (Hui, 1988; Schroeder *et al.*, 1991, 1995; Mattjus *et al.*, 1994; Slotte *et al.*, 1994). In fact, the level of cholesterol in different cellular membranes, and the distribution of cholesterol between different phospholipid vesicles, does appear to depend on the phospholipid composition of the host membrane bilayer (Kudchokar *et al.*, 1983; Yeagle and Young, 1986; Yeagle, 1985, 1988; Slotte and Bierman, 1987; Bittman, 1993; Lange and Steck, 1994; Slotte, 1995). Thus, differences in the relative affinity of cholesterol for the different lipid components of the various membranes, as well as the existence of cholesterol-rich and -poor phospholipid domains, may play an important role in the mechanism(s) which control the steady state distribution of cholesterol within the different membranes of eukaryotic cells (Yeagle and Young, 1986; Yeagle, 1988; van Meer, 1993; Slotte *et al.*, 1994; Liscum and Underwood, 1995).

The Effect of Cholesterol on Biomembrane Structure and Function.

Function of cholesterol in biomembranes

It is clear that animal cells expend considerable amounts of energy to obtain cholesterol and to maintain a particular distribution of this molecule within the various membranes of the cell, but for what purpose? There are two generally accepted roles for cholesterol in biomembranes. The first is the passive modulation of the bulk physical properties of the host membrane. This is especially important for eukaryotic cell plasma membranes, where the majority of the cellular cholesterol is located (Yeagle, 1985, 1988; Bloom *et al.*, 1991). Generally, the incorporation of cholesterol increases the thickness and mechanical strength of the bilayer while also reducing its permeability. Cholesterol also indirectly modulates the activity of membrane proteins via bulk changes in the fluidity of the host membrane, as well as buffeting the cell against large changes in temperature (Paphadjopoulos *et al.*, 1971; McElhaney *et al.*, 1973; Demel and de Kruijff, 1976; Yeagle, 1985, 1988, 1991; Dahl and Dahl, 1988; Bloom *et al.*, 1991; George and McElhaney, 1992; Fielding, 1992; Thewalt and Bloom, 1992). The ability of cholesterol to achieve these effects, without solidifying the membrane or dramatically decreasing lipid mobility, is believed to be a direct consequence of the unique structure of cholesterol since most structural alterations result in a loss in the ability of cholesterol to produce its characteristic effects on lipid bilayers or support growth in sterol-auxotrophic mycoplasma and mammalian cells (Demel and de Kruijff, 1976; Yeagle, 1988; Dahl and Dahl, 1988; Thewalt and Bloom, 1992; McElhaney, 1992a,b). The second role of cholesterol is the direct modulation of the activity of membrane proteins (Yeagle, 1988; Dahl and Dahl, 1988). There are an increasing number of examples for direct cholesterol-protein interactions in membranes, with subsequent modulation of protein activity. The best characterized examples of direct cholesterol-protein interactions are the band III anion transport protein in erythrocytes (Klappauf and Schubert, 1977; Muhlebach and Cherry, 1982), glycophorin (Yeagle, 1984), Na^+/K^+-ATPase (Yeagle *et al.*, 1988; Vermuri and Philipson, 1989), adenylate cyclase (Whetton *et al.*, 1983), the transferrin receptor (Nunez and Glass, 1982), the nicotinic acetylcholine receptor (Narayanaswami *et al.*, 1993; Fernandez-Ballester *et al.*, 1994), Ca^{2+}-ATPase (Ding *et al.*, 1994), human serum amyloid A (Liang *et al.*, 1995), caveolin (Murata *et al.*, 1995), and the oxytocin receptor (Klein *et al.*, 1995). However, it is important to note that in each case it is difficult to unequivocally demonstrate that the bulk effects of cholesterol on the physical properties of the host membrane do not account for differences in protein activity or function (Cheng *et al.*, 1986; Yeagle, 1988, 1989; Fernandez-Ballester *et al.*, 1994; Ding *et al.*, 1995; Mosior *et al.*, 1996).

In summary, cholesterol performs a number of different functions in biological membranes. While cholesterol-phospholipid and cholesterol-protein interactions are both important aspects of the function of cholesterol in biological membranes, the work described in this thesis focuses on the nature of cholesterol-phospholipid interactions. Thus, the following sections introduce the study of cholesterol-phospholipid interactions, and the overall objectives of our investigation.

Cholesterol-phospholipid interactions

During the course of defining the structure, distribution and function of cholesterol in biomembranes, a number of important questions have been raised. What is the relationship between the structure of cholesterol and its unique effect on the physical properties of neighboring lipids? Does cholesterol exhibit phospholipid-specific interactions? If so, could this modulate the distribution between different membranes? Moreover, it is unclear whether cholesterol exerts its characteristic effects on the host membrane bilayer in a random manner, as part of an ordered matrix, or in laterally phase-separated cholesterol-rich domains. Without exception, answers to these questions require detailed knowledge of cholesterol-phospholipid interactions. However, in the complex and dynamic environment of biomembranes, extracting information on cholesterol-phospholipid interactions over various time scales is exceedingly difficult (McElhaney, 1982; Gennis, 1989; Yeagle, 1985, 1992; Bloom and Thewalt, 1995). Thus, to address the fundamentals of cholesterol-phospholipid interactions, investigators have typically employed binary cholesterol/DPPC or cholesterol/DMPC model liposomes in which the composition of the system, as well as its morphology, are tightly controlled. Prior studies of cholesterol-PC interactions in liposomes span almost 30 years and involve a significant number of different techniques, including calorimetry, dilatometry, fluorescence polarization, neutron scattering, X-ray diffraction and ^{1}H-, ^{2}H-, ^{13}C-, ^{31}P-NMR and electron paramagnetic and FTIR spectroscopy (Demel and de Kruijff, 1976; Yeagle, 1985, 1988; Vist and Davis, 1990). Consequently, there is an large body of knowledge derived from studies of model cholesterol/PC bilayers. Based on these studies, Thewalt and Bloom (1992) recently proposed that cholesterol-PC interactions served as an adequate model for cholesterol-phospholipid interactions in biological membranes (see also Ipsen *et al.*, 1987; Vist and Davis, 1990). Specifically, these authors indicate that cholesterol generates a unique liquid-ordered phase in PC membranes, and that the properties of this liquid-ordered phase are supposed to represent of the phase state of biological membranes, regardless of the lipid composition of the host membrane (Ipsen *et al.*, 1987; Bloom *et al.*, 1991; Thewalt and Bloom, 1992). However there is also a small, but significant, body of evidence which suggests that cholesterol-phospholipid interactions may vary markedly with the headgroup or hydrocarbon chain composition of the host phospholipid bilayer (Demel *et al.*, 1977; van Dijck *et al.*, 1979; Blume, 1980; Blume and Griffin, 1982; Bach, 1984; Bach and Wachtel, 1989; Keough *et al.*, 1989; Singer and Finegold, 1990; Kariel *et al.*, 1991; Bach *et al.*, 1992; Cheetham *et al.*, 1992; Borochov *et al.*, 1995). Unfortunately, these studies are not systematic and there are serious discrepancies in the results and conclusions of many of these studies. As a result, there is no model for cholesterol-phospholipid interactions which satisfactorily accounts for variations in the structure of the phospholipid molecule. To develop such a model would require a systematic study of cholesterol-phospholipid interactions in which the structure of the phospholipid polar headgroup and hydrocarbon chains, as well as the cholesterol molecule, are systematically varied.

OVERALL THESIS OBJECTIVE

TO DETERMINE HOW THE STOICHIOMETRY AND NATURE OF CHOLESTEROL-PHOSPHOLIPID INTERACTIONS VARY WITH CHANGES IN THE STRUCTURE OF THE HOST PHOSPHOLIPID BILAYER.

SPECIFIC QUESTIONS

i) Does the nature and magnitude of the effect of cholesterol on the thermotropic phase behavior and organization of the host phospholipid vary with hydrocarbon chain length or unsaturation?

ii) What is the contribution of the alkyl side chain of cholesterol to cholesterol-phospholipid interactions?

iii) How do changes in the structure of the phospholipid polar headgroup affect the nature and magnitude of the effect of cholesterol on the thermotropic phase behavior and organization of the host phospholipid bilayer?

iv) Can we extend our knowledge of cholesterol-phospholipid interactions obtained from model membrane systems to cholesterol-lipid interactions in a biological system?

Experimental methodology

Using a combination of calorimetry and FTIR and ^{31}P-NMR spectroscopy, we have examined the thermotropic phase behavior and organization of cholesterol-containing model phospholipid bilayers varied with respect to the structure of the phospholipid headgroup and hydrocarbon chains. We also examined how changes in the length and structure of the cholesterol C17 side chain altered cholesterol-phospholipid interactions.

Our selection of phospholipids was chosen to cover the major phospholipid headgroup species of eukaryotic cells with significant variations in the lengths of the phospholipid hydrocarbon chains. We have also examined the effect of cholesterol on the thermotropic phase behavior of the total *A. laidlawii* membrane lipids, as well as the membrane lipid components individually. The ability to manipulate lipid fatty acid composition and cholesterol content, and thus to alter phase state and fluidity of the *A. laidlawii* B membrane lipid bilayer, has made this organism an excellent system for the study of cholesterol-lipid interactions in biological membranes (McElhaney, 1992a). Overall, we have systematically examined the effect of cholesterol, or a related sterol, on the thermotropic phase behavior and organization of 48 different host lipid bilayers, a greater number of binary systems than all previous investigations of cholesterol-phospholipid interactions combined. More importantly, our systematic approach to the study of the different phospholipid and glycolipid species, varying both hydrocarbon chain length and structure and polar headgroup composition, has allowed us to unequivocally determine that cholesterol-lipid interactions are specific for the composition of lipid polar headgroup and hydrocarbon chains. Since model membranes and DSC, FTIR and ^{31}P-NMR spectroscopy are central to the work presented in this thesis, a brief description of these techniques and the phase behavior of aqueous lipid dispersions precedes the presentation of the results.

Lipid phase behavior and model bilayer membranes

In water, phospholipids exhibit extremely low critical micellar concentrations and will aggregate into larger assemblies to limit the contact of the hydrocarbon chains with water (Singer and Nicholson, 1972; Gennis, 1989; Isrealachvili, 1992). Membrane phospholipids are polymorphic, thus they can exist in a number of different organized structures when dispersed in an aqueous milieu (Luzatti and Tardieu, 1974; Gruner *et al.*, 1985; Gennis, 1989; Lewis and McElhaney, 1992; Gruner, 1992). The polymorphic forms of an individual lipid depend on the molecular shape of the lipid which is approximated by the relative cross-sectional areas of the headgroup and of the hydrocarbon chains (Gennis, 1989; Kumar, 1991). Changes in the size of the headgroup or the degree of unsaturation in the lipid hydrocarbon chains can have dramatic consequences on lipid interactions and the type of lipid aggregate formed which include, but are not limited to, micelles, lamellar bilayers or inverted hexagonal phases. The particular polymorphic form of a given phospholipid also depends on temperature, headgroup hydration, pressure, pH, ionic strength, or even the presence of cholesterol (Gennis, 1989; Isrealachvili, 1992). Despite the plethora of factors which may favor different phospholipid aggregate structures, the biomembranes of eukaryotic cells are uniformly bilayer, and as yet no other forms have been identified *in vivo*. However, the relative amounts of bilayer-preferring and non-bilayer-preferring lipids may be biosynthetically regulated in response to variations in temperature, and bilayer composition (Gennis, 1989; Gruner, 1992). Lastly, it is important to note that cholesterol itself does not form bilayers when dispersed in water but will form either dehydrated or monohydrate crystals in solution in the absence of phospholipids (Loomis *et al.*, 1979).

The large majority of phospholipids which comprise biological membranes, singularly and in mixtures, also undergo thermally induced, lamellar gel to liquid-crystalline phase transitions under physiological conditions (Lewis and McElhaney, 1992). Thus, the gel to liquid-crystalline phase transition has been studied extensively in order to better understand the physical chemistry of membranes. In this transition the phospholipid hydrocarbon chains are converted from a relatively rigid, extended all-*trans* conformation in the gel state to a more orientationally disordered state characterized by *gauche* conformers and increased rates of intra- and intermolecular motions (Lewis and McElhaney, 1992). The phase transition is accompanied by a pronounced lateral expansion with a concomitant decrease in bilayer thickness and a small increase in volume. The phase transition is characterized as a pseudo-first order transition occurring over a relatively small temperature range (< 1 °C) for the pure phospholipid and is endothermic on heating and exothermic on cooling. Pure lipids also often exhibit gel-state polymorphism and thus undergo gel/gel transitions which do not involve chain melting. Since the temperature, enthalpy and cooperativity of gel/liquid-crystalline and gel/gel phase transitions are acutely sensitive to the structure(s) of the lipid(s) which comprise the bilayer, studies of these transitions in different lipids and lipid mixtures have provided a wealth of information on how the lipid-lipid and/or cholesterol-lipid interactions contribute to differences in the phase state and organization of lipid bilayers.

It is important to note that the ionization state and degree of hydration of different phospholipids or glycolipids in fully hydrated bilayers may vary markedly with changes in the pH or ionic strength of the lipid/water mixture (Cevc and Marsh, 1987; Lewis and McElhaney, 1993). Changes in the ionization state or hydration of a given phospholipid or glycolipid species in turn can have a dramatic effect on the temperature and enthalpy of the lamellar gel/liquid-crystalline or lamellar/non-lamellar phase transitions of the lipid bilayer. Furthermore, the presence of appreciable levels of certain divalent cations may also induce isothermal phase transitions in bilayers composed of the acidic phospholipids (Cevc and Marsh, 1987; Lewis and McElhaney, 1993). To ensure that variations in pH did not influence the results of our study, we hydrated the cholesterol/phospholipid and cholesterol/glycolipid mixtures in deionized water containing 50 mM Tris (high-sensitivity DSC and ^{31}P-NMR spectroscopy) or 50 mM $NaHPO_4$ (FTIR spectroscopy) with the pH adjusted to approximately 7.2. In addition, each of the buffered solutions contained 10 mM EDTA to chelate divalent cations as well as 100 mM NaCl to provide a constant level of electrostatic screening (Cevc and Marsh, 1987). With this protocol we are confident that the ionization state and degree of hydration of the different phospholipids and glycolipids, with or without cholesterol, did not vary significantly from that observed under physiological conditions. Thus we eliminated the possibility of pH-, salt- or divalent cation-induced shifts in the temperature and enthalpy of the phase transitions of the lipid or cholesterol/lipid mixtures examined in this study.

Techniques for the study of the thermotropic phase behavior and organization of model and A. laidlawii B membranes

i) *Differential scanning calorimetry*

DSC has proven an exceptionally valuable technique in the study of lipid phase transitions. In a DSC instrument there are two cells, the sample cell with the lipid suspension in buffer, and the reference cell with buffer only. Both cells are heated at an identical rate while the temperature of the cells is monitored continuously. If the heat capacity of the sample and reference are equal, there is no temperature difference between the two; however, over the temperature range of a phospholipid chain melting transition, the heat capacity of the sample cell increases dramatically, thus more heat is required to maintain the zero temperature difference between reference and sample cell. This energy is recorded as the excess specific heat capacity as a function of temperature. The resulting curve provides the thermodynamic parameters associated with the transition in which: (i) the T_m refers to the phase transition temperature where the excess specific heat capacity reaches a maximum; (ii) the $\Delta T_{1/2}$ represents the intermolecular cooperativity of the phase transition (defined by the van't Hoff enthalpy H_{vH}, approximated $6.9T_m^2/\Delta T_{1/2}$, where a completely cooperative, first order transition exhibits H_{vH} approaching infinity, a non-cooperative transition, unity), and; (iii) ΔH represents the enthalpy of the transition (determined by the area under the curve) which is usually expressed in kcal/mol.

One of the useful features of phospholipid phase transitions is that the temperature, enthalpy and cooperativity of the chain-melting transition of pure phospholipid bilayers is acutely sensitive to additives. Even with as little as 0.5 mol%, additives have a significant impact on the phase transition temperature, cooperativity and enthalpy of the phase transition of the host phospholipid bilayer when monitored using high-sensitivity DSC instruments (McElhaney, 1982; Biltonen and Lichtenberg, 1993). This has proven particularly useful when examining cholesterol-phospholipid interactions, as is outlined later. Moreover, changes in the cooperativity and temperature of the main chain melting transition of complex phospholipid mixtures may also be used to establish the relative miscibility of the components, to monitor the formation of stoichiometric complexes, or to identify lateral or full scale phase separation (Lewis and McElhaney, 1992). Finally, high-sensitivity DSC provides the most accurate measure of the thermodynamics of a phase transition and it does not require the use of probes which may influence the thermotropic phase behavior of the phospholipid bilayer. However, high-sensitivity DSC experiments must be performed when the mixture(s) are at equilibrium. This can be difficult to ascertain since calorimetry does not directly provide information on the organization or phase state of the phospholipid mixture (McElhaney, 1982).

ii) *Fourier transform infrared spectroscopy*

FTIR spectroscopy is a non-perturbing technique which is used to directly determine the conformational state and organization of phospholipid bilayers. FTIR is based on the vibrational modes of particular chemical moieties which, upon absorption of radiation at a given frequency, will undergo a transition between two vibrational states. The transitions of different chemical bonds exhibit unique spectral band frequency maxima. In addition, changes in the conformation or environment also affect the absorption frequency and bandwidth; however, the frequency range of a particular chemical group in various environments or conformations is usually within 100 cm^{-1}. With the advent of fast Fourier transform algorithms and data handling protocols such as solvent subtraction and band deconvolution, FTIR is now widely utilized to study lipid conformation in membranes over a wide range of temperatures in the presence of excess H_2O or D_2O (Casal and Manstch, 1984; O'Leary, 1992). In solution FTIR experiments, a number of different bands function particularly well as spectroscopic probes in phospholipid bilayers, which are conveniently located in the headgroup region (O=P-O- stretching at 1250 cm^{-1} and choline C-N stretching at 717 cm^{-1}), at the interface (C=O stretching at 1700 to 1800 cm^{-1}), and the in the hydrophobic core of the bilayer (*sn*-1 and *sn*-2 hydrocarbon chain CH_2 stretching bands at 2800-3000 cm^{-1} and CH_2 scissoring band at 1450-1480 cm^{-1}) (Casal and Manstch, 1984; Mendelsohn and Mantsch, 1986; Mantsch and McElhaney, 1991; Lewis and McElhaney, 1992, 1996). Thus, FTIR is well suited to examine the organization of phospholipid bilayers and their thermotropic polymorphism. Changes in the curve shape, width and maxima of the CH_2 stretching and scissoring bands reflect changes in the conformation and lateral packing of the phospholipid hydrocarbon chains with temperature or upon the addition of an additive such as cholesterol. Similarly, the O=P-O and C=O stretching regions each exhibit characteristic absorption maxima and bandwidths for different crystalline and gel states which coincide with changes in interfacial orientation, mobility and hydrogen bonding of phospholipids in the gel and crystalline states or with changes in temperature (Lewis and McElhaney, 1992, 1996). Thus, the ability to monitor a number of characteristic bands simultaneously has permitted investigators to unambiguously identify crystalline, gel and liquid-crystalline phases in phospholipid bilayers and to correlate this information with HS-DSC studies of the temperature and energetics of transitions between these states (Mantsch and McElhaney, 1991; Lewis and McElhaney, 1993, 1996). It is also important to note that the timescale of these measurements is extremely short, on the order of 10^{-12} sec-1. Thus FTIR can sample acyl-chain *trans-gauche* rotational isomerization without interference from longer timescale motions such as lipid rotation or diffusion, while other methods, such as ^{2}H-NMR, require elaborate models and subtraction protocols to account for these slower motions (Davis, 1983; Mendelsohn and Mantsch, 1986; Blume, 1988; Gennis, 1989). In summary, the information on the structure and organization of lipid bilayers from FTIR is an excellent complement to the energetic information on the phase behavior of lipid or cholesterol/lipid mixtures obtained from DSC.

iii) *Nuclear magnetic resonance (^{31}P)*

^{31}P-NMR spectroscopy has made a significant contribution to our understanding of the dynamics of phospholipid bilayers. Briefly, NMR spectroscopy utilizes the quantized properties of nuclear spins, which, in the presence of an applied magnetic field, will provide a net magnetization. A transient radiofrequency pulse is then used to induce a net transition between the quantized spin states, which will then decay back to equilibrium over time. From the relaxation processes, information on both molecular order and motional rates can be derived, and in some cases additional information regarding molecular orientation and anisotropy of molecular motion can also be obtained (Seelig, 1977, 1980; Davis, 1983). In model membranes, the use of ^{31}P-NMR has been used primarily to examine the polymorphism of phospholipid mixtures. The formation of inverted hexagonal, lamellar or cubic phospholipid phases all provide distinct ^{31}P-NMR powder patterns (Seelig, 1978; Cullis and de Kruijff, 1978). ^{31}P-NMR can also be used to determine the phase state of the phospholipid bilayer. In proton decoupled ^{31}P-NMR spectra of unsonicated liquid-crystalline lamellar bilayers, basal linewidths of approximately 50-75 ppm are observed, representing the fast, axially symmetric motion of the phosphorous of the phospholipid headgroups (Seelig, 1978; Campbell *et al.*, 1979). In the gel state the basal spectral linewidths approach 100 ppm and in the crystalline phase, where ^{31}P nuclei undergo long lived intermolecular hydrogen bonds, the basal linewidth approaches 250 ppm and the intensity of the spectrum degrades markedly (Lewis and McElhaney, 1993). Overall, the information on the morphology of phospholipid or cholesterol/phospholipid mixtures at different temperatures, as well as the relative ease with which ^{31}P-NMR data can be obtained, were primary considerations in our choice of ^{31}P-NMR to complement our DSC and FTIR analysis of particular cholesterol-containing phospholipid bilayers.

Figure I-1. Full generalized structure of the dominant sterols of most eukaryotic organisms.

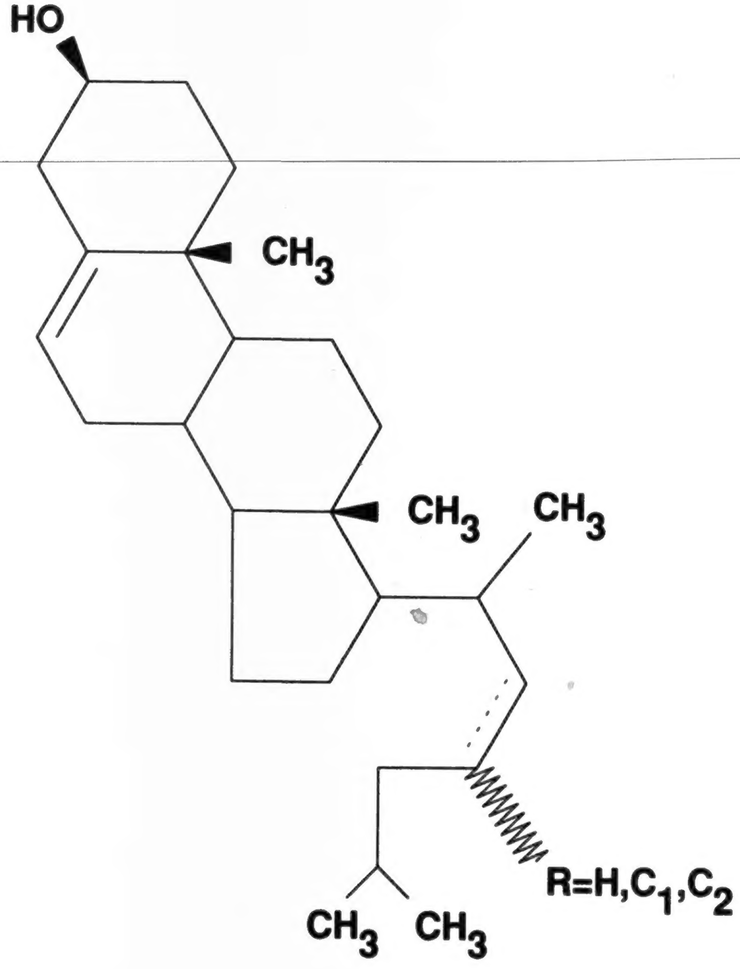
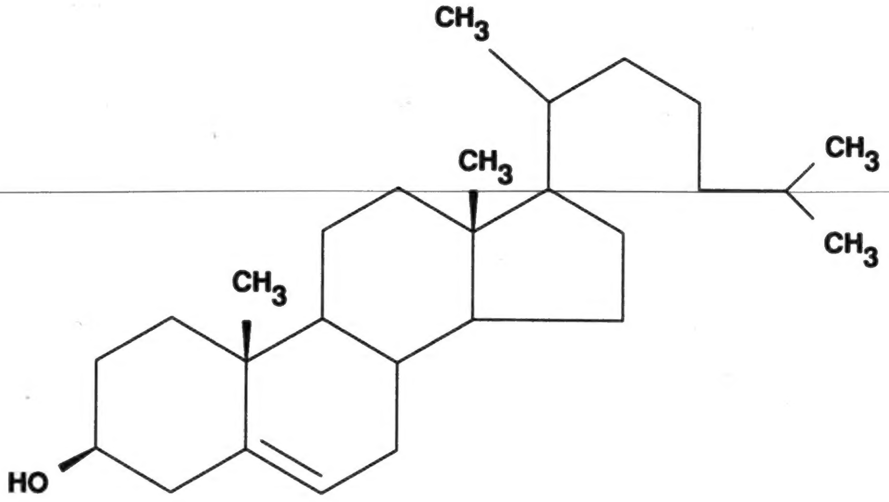

HO
CH3
CH3
CH3
R=H,C1,C2
CH3
CH3

Figure I-2. Structure of the cholesterol molecule, the dominant sterol in higher eukaryotes.


CH3
CH3
CH3
CH3
CH3
HO

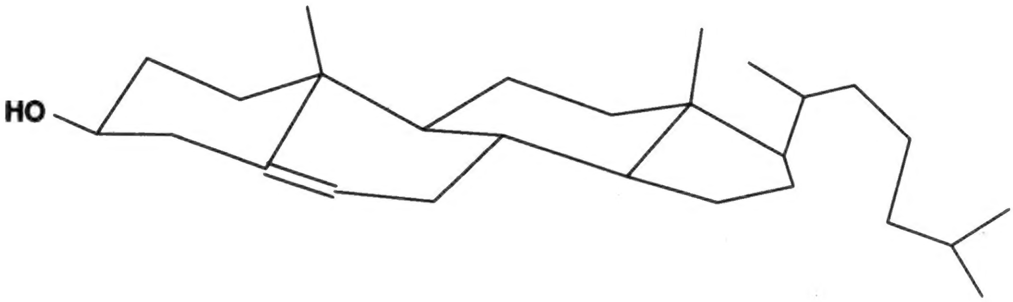
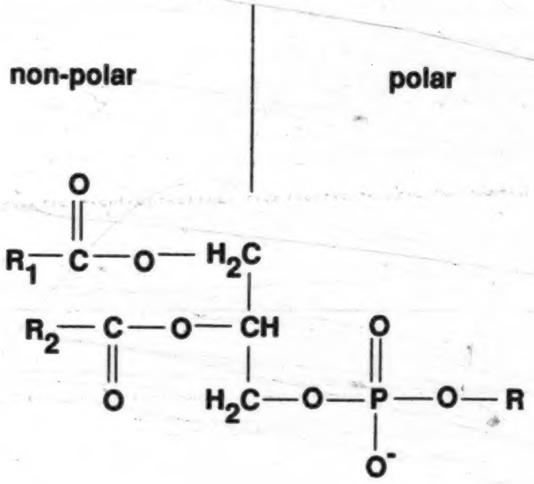

HO

Figure I-3. Representative structures for selected phospholipid molecules. Note, for the purpose of this thesis, the stereospecific numbering scheme is used to describe the phospholipids in which the phospholipids are derivatives of *sn*-glycero-3-phosphoric acid (Hirschmann, 1956).

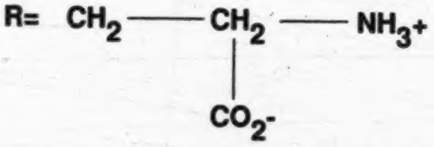


Phosphatidic acid $R= H$

Phosphatidylcholine $R= CH_2 - CH_2 - N^+(CH_3)_3$

Phosphatidylethanolamine $R= CH_2 - CH_2 - NH_3^+$

Phosphatidylserine



$$R= CH_2 - \underset{\substack{|\\ CO_2^-}}{CH_2} - NH_3^+$$

Table I-1. Lipid compositions of subcellular organelles from rat liver. Values in weight% except for cholesterol, where values are presented as a mole fraction of the total phospholipids (Adapted from White, 1973 and Ansell and Spanner, 1982).

	Phospholipids						
	PC	PE	PS	PI	SPM	CL	CHOL
Organelle							
Endoplasmic reticulum	56%	21%	4%	9%	4%	2%	0.10
Mitochondria	42%	32%	4%	5%	2%	15%	0.08
Lysosome	42%	20%	0%	6%	16%	0%	0.45
Nuclear membrane	52%	25%	6%	4%	6%	0%	0
Golgi membrane	45%	17%	4%	9%	12%	0%	0.10
Plasma membrane	43%	20%	4%	7%	23%	0%	0.85

Table I-2. Transbilayer lipid asymmetry in human erythrocyte and rat liver plasma membranes. Values in weight %. (Adapted from White, 1973 and Ansell and Spanner, 1982).

	Human erythrocyte PM		Rat liver PM	
	% Outer leaflet	% Inner leaflet	% Outer leaflet	% Inner leaflet
PC	80%	20%	75%	25%
PE	18%	80%	48%	52%
PS	0%	100%	0%	100%
SM	85%	15%	65%	35%

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CHAPTER II. DIFFERENTIAL SCANNING CALORIMETRIC STUDY OF THE EFFECT OF CHOLESTEROL ON THE THERMOTROPIC PHASE BEHAVIOR OF A HOMOLOGOUS SERIES OF LINEAR SATURATED PHOSPHATIDYLCHOLINES[2]

INTRODUCTION

The occurrence of high concentrations of cholesterol in the plasma membranes of higher organisms has prompted numerous investigations into its role in the structure and function of cell membranes (for reviews, see Dahl and Dahl, 1988; Yeagle 1988). Although cholesterol appears to have several different functions in cells, one of its primary roles is as a modulator of the physical properties of the plasma membrane phospholipid bilayer. Thus a large number of studies of the interactions of cholesterol with phospholipid monolayers and bilayers have been carried out utilizing a wide range of physical techniques (for reviews, see Demel and de Kruijff, 1976; Razin and Rottem, 1978; Yeagle, 1985, 1988; Finean 1990). Some of the major effects of cholesterol incorporation observed in such studies include: i) a broadening and eventual elimination of the cooperative gel to liquid-crystalline phase transition of the phospholipid bilayer; ii) a marked increase (decrease) in the orientational order of the phospholipid hydrocarbon chains above (below) the phase transition; iii) a decrease in the phospholipid acyl chain tilt angle in the gel phase; and iv) the abolition of the phospholipid pretransition at low cholesterol contents. In addition, cholesterol decreases (increases) the passive permeability of liquid-crystalline (gel) phospholipid bilayers and condenses (expands) the fluid (solid) state of phospholipid monolayers, as well as altering the mechanical properties of the phospholipid bilayers. Despite this impressive body of experimental data, our understanding of the molecular basis of cholesterol/phospholipid interactions remains incomplete.

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Differential scanning calorimetry is a thermodynamic technique which has proven of great value in studies of lipid thermotropic phase behavior in model phospholipid bilayers and biological membranes (Mabrey and Sturtevant, 1976; McElhaney, 1982, 1984, 1989; Keough, 1984). A considerable number of DSC studies of cholesterol/phospholipid binary mixtures have been carried out over the last twenty-five years, by far the majority on cholesterol/DPPC or, to a lesser extent, cholesterol/DMPC systems. Although all earlier studies, which used relatively low-sensitivity calorimeters, agreed that the progressive addition of cholesterol broadens the gel to liquid-crystalline phase transition of these phospholipids and progressively reduces the transition enthalpy, there was considerable disagreement about the details of this interaction. For example, the addition of cholesterol was reported to either considerably reduce (Ladbrooke *et al.*, 1968), to have little effect on (Hinz and Sturtevart, 1972; Gershfeld 1978) or to increase (Calhoun and Shipley, 1979) the phospholipid phase transition temperature. Also, the cooperative phospholipid phase transition was reported to be completely abolished at cholesterol concentrations ranging from 33 to 43 mol%. Similar results were reported for chemical analogues of DPPC (De Kruyff *et al.*, 1973) and for the interaction of cholesterol with various binary mixtures of linear saturated PC's (De Kruyff *et al.*, 1974). Moreover, only a single endothermic transition was detected in all of these studies at all cholesterol concentrations studied.

More recent high-sensitivity DSC studies of cholesterol/PC interactions, however, have revealed a more consistent but also more complex picture of cholesterol/DPPC and cholesterol/DMPC interactions (Estep *et al.*, 1978; Mabrey *et al.*, 1978; Genz *et al.*, 1986; Vist and Davis, 1990). At cholesterol concentrations from 0 to 20-25 mol%, the DSC endotherm consists of two components. The sharp component exhibits a phase transition temperature and cooperativity only slightly reduced from that of the pure phospholipid, and the enthalpy of this component decreases linearly with increasing cholesterol content, becoming zero at 20-25 mol%. In contrast, the broad component exhibits a progressively increasing phase transition temperature and enthalpy and a progressively decreasing cooperativity over this same range of cholesterol content. Above cholesterol levels of 20-25 mol%, the broad component becomes progressively less cooperative, the phase transition midpoint temperature continues to increase, and the transition enthalpy continues to decrease, eventually approaching zero only at cholesterol concentrations near 50 mol%. These results suggest that at low cholesterol concentrations, cholesterol-poor and cholesterol-rich domains coexist, with the former decreasing in proportion to the latter as cholesterol concentrations increase. In fact, a cardinal point in the cholesterol/DPPC phase diagram at about 22 mol% had been predicted from earlier model building studies of Engleman and Rothman (1972), who calculated that the cholesterol molecule could interact with a maximum of seven adjacent phospholipid hydrocarbon chains (or 3.5 phospholipid molecules) and thus that free phospholipid would exist only at cholesterol concentrations below this value. This model also explains the decreasing enthalpy of the broad component observed above 22 mol% cholesterol, since an increasing proportion of phospholipid molecules would interact with more than one cholesterol molecule rather than with the more flexible hydrocarbon chains of adjacent phospholipids, thus progressively decreasing and eventually abolishing the cooperative chain-melting phase transition. However, this model may not be valid for all PC's, since DSC studies of PC's containing one or two unsaturated fatty acyl chains can apparently exhibit quite different cholesterol/PC stoichiometries (Davis and Keough, 1983; Keough *et al.*, 1989; Kariel *et al.*, 1991).

The only systematic DSC study on the effect of variations in bilayer thickness on cholesterol/phospholipid interactions is that of Singer and Finegold (1990). In studying the effect of increasing cholesterol levels on linear saturated PC's containing 12-20 carbon atoms, these investigators reported that the enthalpy of the main transition of all these phospholipids decreased with increasing cholesterol levels as reported previously. However, they also reported the rather surprising finding that the cholesterol concentration required to reduce the transition enthalpy to zero also increases linearly with the length of the PC hydrocarbon chains. We believe that the validity of this latter observation is suspect for several reasons. First, these workers report that cholesterol concentrations of only about 17 and 26-27 mol% are sufficient to completely abolish the cooperative main transitions of DMPC and DPPC, respectively, whereas all recent high-sensitivity DSC studies find that a cooperative phase transition persists until cholesterol concentrations reach 50 mol%. Second, a linear and marked dependence of cholesterol/PC interaction stoichiometry on hydrocarbon chain length can not be explained by any of the current models of cholesterol/PC interactions. Finally, the use of a low-sensitivity DSC instrument with constant and relatively small phospholipid samples raises the possibility that the less energetic and less cooperative phase transitions, characteristic particularly of the shorter chain length PC's and higher cholesterol concentrations, will not be detected. For these reasons, we have reinvestigated the effect of the incorporation of increasing quantities of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated PC's, utilizing high-sensitivity DSC and an experimental protocol ensuring that broad, low enthalpy phase transitions can be accurately monitored. Indeed, using such an approach, we find that the amount of cholesterol required to completely abolish the cooperative chain-melting phase transition of all PC's studies is 50 mol%, regardless of hydrocarbon chain length. Moreover, we also demonstrate that the phase transition temperature of the broad component of the DSC endotherm shifts either to lower or higher temperatures in accordance with the direction and degree of mismatch between the thickness of the hydrophobic core of the PC bilayer and the length of the cholesterol molecule.

MATERIALS AND METHODS

The PC's in this experiment were synthesized in this laboratory by methods previously shown to ensure highly pure samples (Lewis and McElhaney, 1985). The cholesterol was purchased from Fisher chemicals and recrystallized twice from ethanol before use. Both cholesterol and the PC's were dissolved in chloroform and mixed from those stock solutions. Mixtures were then dried under N_2 and evaporated to dryness in a vacuum overnight. The dried mixtures were then dispersed and suspended with deionized water, heated to approximately 10-20 °C above the phase transition temperature of the PC studied, and then vortexed to give a multilamellar suspension. The amounts of PC used varied from 0.5 to 2.0 mg for pure lipid samples to 10-12 mg for samples containing high concentrations of cholesterol. The calorimetric analysis was performed on a high-sensitivity instrument manufactured by Hart Scientific. Transition signal strength was also enhanced via scan rate variation depending on cholesterol concentration. Scan rates as low as 5°C/hr were used for pure lipid samples, and then progressively increased to 55 °C/hr for samples approaching 50 mol% cholesterol. This experimental protocol ensured that the broad, low-enthalpy endotherms observed at high cholesterol concentrations, particularly with the shorter chain PC's, are accurately recorded. Sample runs were repeated at least three times and as many as 15 times to ensure reproducibility. After calorimetric analysis, quantification of the phospholipids was achieved by gas chromatographic analysis of the PC fatty acid methyl esters after transesterification with acidic methanol using a known amount of appropriate PC as an internal standard. The methyl esters were quantified as described previously (Lewis and McElhaney, 1985). Cholesterol quantification was done using the assay developed by Watson (1960). Thin-layer chromatography was used to check if sample degradation had occurred during calorimetric analysis. No degradation was found in any of the samples.

RESULTS

Thermotropic phase behavior of pure phosphatidylcholines

Representative high-sensitivity DSC scans for a typical member of the PC homologous series studied, DPPC, are presented in Figure II-1 as a function of increasing cholesterol content. In the absence of cholesterol, unannealed DPPC bilayers exhibit two endotherms on heating, a lower temperature, lower enthalpy pretransition and a higher temperature, higher enthalpy main transition. The pretransition arises from the conversion of a lamellar gel (L_{β}') phase to the rippled gel (P_{β}') phase and the main phase transition from a conversion of the P_{β}' phase to the lamellar liquid crystalline (L_{α}) phase. Since the pretransition increases in temperature more steeply with increasing hydrocarbon chain length than does the main transition, the temperature interval between these two events decreases as the length of the PC chains increase. Thus for 21:0 PC, a discrete pretransition is not observed and the main transition results from a L_{β}'/L_{α} conversion. Similarly, 13:0 PC (and 12:0 PC, not studied here) are unique in this homologous series of

PC's in not exhibiting a pretransition but in exhibiting instead a higher temperature shoulder on the main phase transition endotherm. Although the physical basis of this behavior is not fully understood, both thermal events are known to involve chain melting (Morrow and Davis, 1987; Lewis and McElhaney, unpublished observations) and will thus be considered together to constitute the main phase transition in the following analyses. For a more thorough discussion of the thermotropic phase behavior of the linear saturated PC series, the reader is referred to Lewis *et al.* (1987) and to the references cited therein.

The effect of cholesterol on the pretransition

The DSC scans presented in Figure II-1 suggest that the pretransition of DPPC is abolished at cholesterol concentrations between 5 and 10 mol%. In order to illustrate the effect of cholesterol on the pretransition more clearly, in Figure II-2 we present a series of optimized DSC scans of DPPC containing 0-6 mol% cholesterol. From these scans it is clear that cholesterol incorporation progressively decreases the pretransition temperature and enthalpy in an approximately linear manner without significantly altering the cooperativity, and that the pretransition becomes undetectable at a cholesterol concentration of 6 mol%. Similar results were obtained for all of the other linear saturated PC's (14:0-20:0 PC) exhibiting discrete pretransitions (data not presented). Thus the effect of cholesterol on the pretransition is not dependent on the hydrocarbon chain length of the host PC bilayer.

The effect of cholesterol on the main phase transition

The DSC scans in Figure II-1 illustrate the overall effect of the incorporation of increasing quantities of cholesterol on the DPPC main phase transition. As presented in Figure II-3, the asymmetric DSC endotherms observed at cholesterol concentrations of 1-20 mol% (5-15 mol% shown) can be well fit by deconvolution into two components. The phase transition temperature of the sharp component decreases slightly with cholesterol incorporation (Figure II-4), and its cooperativity decreases moderately. Moreover, as illustrated in Figure II-5, the enthalpy of the sharp component of DPPC decreases linearly with increases in cholesterol content and becomes zero between 20-25 mol%. Similar behavior was exhibited by all chain lengths of PC investigated. In contrast, the phase transition temperature of the broad component for DPPC increases fairly markedly with increasing cholesterol incorporation, and its cooperativity decreases markedly. Thus, the broad component encompasses a very large temperature range at high cholesterol levels. As shown in Figure II-6, the enthalpy of the broad component of DPPC first increases with increases in cholesterol incorporation to a level of 20-25 mol%, and then decreases to zero at cholesterol concentrations near 50 mol%. Again, similar behavior is noted for all the other PC's studied.

In order to compare directly our results with those of Singer and Finegold (1990), in Figure II-7 we have plotted the overall enthalpy of the main phase transition (broad plus sharp component when present), as a function of cholesterol content for representative members of the homologous series of PC's studied. As expected from the analysis of the individual components just presented, the total enthalpy decreases in an approximately linear fashion with increasing cholesterol levels for all the PC's studied. Moreover, in all cases the enthalpy does not approach zero until cholesterol levels of near 50 mol% are reached. In particular, no marked dependence of the apparent cholesterol/PC interaction stoichiometry is evident, in contrast to the results of Singer and Finegold (1990). We thus conclude that, with the exceptions to be discussed below, the overall effects of cholesterol on PC thermotropic phase behavior are qualitatively and quantitatively similar for all members of this homologous series and thus that hydrocarbon chain length, and hence bilayer thickness, is not a major determinant of the nature of cholesterol/PC interactions.

In these studies we did identify two thermodynamic parameters that do appear to be dependent on the hydrocarbon chain length of the PC molecule and these are the phase transition temperature and the cooperativity of the broad component of the main phase transition. As illustrated in Figure II-8, we find that the phase transition midpoint temperature of the broad component is progressively shifted to higher temperatures (relative to the phase transition temperature of the pure PC) as the hydrocarbon chain length decreases below 17 carbons and vice versa as the chain length increases above 17 carbons. Furthermore, this effect is asymmetric in that it is of greater magnitude and is manifest at lower cholesterol concentrations in the shorter chain PC's as compared to the longer chain PC's. The width of the broad component, shown in Figure II-9, also displays a differential response with respect to chain length. At chain lengths <18 carbons, the $\Delta T_{1/2}$ of the broad component increases with increasing cholesterol incorporation more rapidly and to a larger extent than observed for PC's with chain lengths >18 carbons. Although we do not at present fully understand the mechanism behind the chain length-dependent effect of cholesterol on the cooperativity of the main phase transition, we do believe that the characteristic shift in phase transition temperature can be explained on the basis of a mismatch between the thickness of the hydrocarbon core of the PC bilayer and the effective hydrophobic length of the cholesterol molecule (see Discussion).

DISCUSSION

Our finding that cholesterol concentrations of greater than 5 mol% abolish the pretransition of the 14:0-20:0 PC's is in good agreement with previous high-sensitivity studies of DPPC (Mabrey *et al.*, 1978; Genz *et al.*, 1986; and Vist and Davis, 1990) and DMPC (Mabrey *et al.*, 1978), although Estep *et al.* (1978) claimed that as little as 3.6 mol% cholesterol abolishes the pretransition. However, our results clearly show that the pretransition persists until 6 mol% cholesterol. The progressive reduction in the temperature and enthalpy of the pretransition with increasing cholesterol concentration is a new finding and may account for the inability of Estep *et al.* (1978) to detect a discrete pretransition at cholesterol levels above 3 mol%. The abolition of the pretransition by low levels of cholesterol is presumably due to the conversion of the P_{β}' phase of these PC bilayers into an L_{β}-like phase, in which the hydrocarbon chains become more perpendicular to the bilayer plane and the bilayer initially thickens (see Finean, (1990) for further discussion).

Our results on the effect of increasing concentrations of cholesterol on the thermotropic phase behavior of the entire homologous series of linear saturated PC's are also in good agreement with previous high-sensitivity DSC studies of DPPC and DMPC (Estep *et al.*, 1978; Mabrey *et al.*, 1978; Genz *et al.*, 1986; Vist and Davis, 1990) although, unlike Mabrey *et al.* (1978) and Tampe *et al.*, (1991), we did not require three components to fit our DMPC curves at cholesterol concentrations between 10 and 20 mol%. In contrast, our results do not agree with the recent low-sensitivity DSC study of Singer and Finegold (1990), who claim that the amount of cholesterol required to completely abolish the main phase transition of members of the same homologous series of linear saturated PC's varies linearly with chain length from as little as 8.5 mol% for 12:0 PC to as much as 43.3 mol% for 20:0 PC. On the contrary, our data shows that the main phase transition of all the PC's studied persists until cholesterol levels of 50 mol% are reached, regardless of hydrocarbon chain length. Therefore, the stoichiometry of cholesterol/PC interactions seems largely independent of the thickness of the host PC bilayer. Our findings thus support the qualitative applicability of the cholesterol/DPPC phase diagrams derived experimentally (Vist and Davis, 1990; Sankaram and Thompson, 1990a) and theoretically (Ipsen *et al.*, 1987; Sperotto *et al.*, 1989) to other members of the linear saturated PC homologous series.

The explanation for the discrepancy in our DSC results as compared to those of Singer and Finegold (1990) is relatively straightforward. The ability to detect a discrete endothermic event using DSC is limited by the sensitivity of the calorimeter employed and by both the intrinsic enthalpy and the cooperativity of that event. Thus even a low-sensitivity DSC instrument can detect the highly cooperative, relatively energetic gel to liquid-crystalline phase transitions of pure PC's but may be unable to detect broad phase transitions of comparable total enthalpy. Since the incorporation of increasing amounts of cholesterol into a PC bilayer decreases the enthalpy of the main transition linearly and also decreases the cooperativity even more markedly, it is clear that a low-sensitivity DSC instrument may lose the ability to discriminate the broad low-enthalpy phase transition from the baseline as cholesterol concentrations increase, particularly if the amount of PC in the sample is kept constant. This effect will be more pronounced as the chain length of the PC decreases, because the initial enthalpy before cholesterol addition decreases markedly with chain length and because cholesterol decreases the cooperativity of the broad component of the main phase transition more rapidly and to a greater extent as the chain length of the PC decreases (see Figure II-9). For these reasons it can appear that cholesterol abolishes the main phase transition of the shorter chain PC's well before that transition actually disappears. In fact, using a low-sensitivity DSC (a Perkin-Elmer DSC-2C) and the experimental protocol of Singer and Finegold, we can reproduce their results, at least qualitatively. However, if a high-sensitivity DSC instrument is employed with the sample size and scan rate maximized to compensate for the effect of cholesterol on the main transition, as in the present study, then clearly defined (albeit broad and poorly energetic) phase transitions can be detected for all the PC's studied here at cholesterol concentrations approaching 50 mol%.

A new and important finding to arise from this study is the observation of a characteristic shift in the temperature of the broad component main phase transition of cholesterol/PC mixtures, dependent on the length of the phospholipid hydrocarbon chain. This effect was neither seen nor predicted to occur in any prior study or theoretical treatment. The biphasic nature of the temperature shift suggests that cholesterol stabilizes the gel phase of shorter chain PC's (<17:0 PC), while destabilizing the gel phase of bilayers composed of PC's with acyl chains longer than 18:0 carbons. This characteristic shift can be explained, at least qualitatively, by reference to the hydrophobic mismatch between the effective length of the cholesterol molecule compared to that of the PC hydrocarbon chains normal to the bilayer plane (see Mouritsen and Bloom, 1984 and references therein). The key point here is that the hydrophobic thickness of PC bilayers decreases by approximately one-third at the gel to liquid-crystalline phase transition due to the introduction of gauche conformers into the all-*trans* hydrocarbon chains. Thus, the introduction of another type of molecule, for example a transmembrane α-helical peptide also orientated with the long axis perpendicular to the bilayer plane, can effect the relative stability of the gel and liquid-crystalline phases according to the degree of mismatch between the effective hydrophobic length of the peptide and the phospholipid hydrocarbon chains. At least to a first approximation, the relative stability of gel and liquid-crystalline phases, and thus the chain-melting phase transition temperature, should be least affected when the hydrophobic length of the peptide equals the mean hydrophobic thickness of the bilayer (i.e., has a hydrophobic length midway between that of the gel and liquid-crystalline phases). Peptides with a greater hydrophobic length would tend to differentially stabilize the gel phase of the host phospholipid bilayer, thus increasing the phase transition temperature, and vice versa. In fact, there is now good experimental evidence for the existence of this hydrophobic mismatch effect in reconstituted synthetic peptide-PC or synthetic peptide-cholesterol-PC bilayer systems (Zhang *et al.*, 1992; Nezil and Bloom, 1992).

One can apply this analysis to the cholesterol/PC systems studied here to good effect. As a first approximation, we assume that the cholesterol molecule is arranged with the long axis perpendicular to the PC bilayer with the 3-OH at the bilayer interface and that the effective length of the cholesterol molecule is temperature invariant, which is equivalent to assuming that the conformation of the cholesterol alkyl group does not change with temperature but remains fully extended. This leads to an effective hydrophobic length of the cholesterol molecule of about 20 Å. Further assuming for the moment that the presence of cholesterol does not effect the conformation of the hydrocarbon chains in the gel and liquid-crystalline states, one could then calculate that the optimum match should occur between cholesterol and 19:0 PC, which has a mean hydrophobic length of about 19.8 Å. However, there is experimental evidence that the alkyl side chain of the cholesterol molecule does possess some restricted conformational freedom in PC bilayers (Kroon *et al.*, 1975; Opella *et al.*, 1976; Dufourc *et al.*, 1984; Duax *et al.*, 1988). If this is so, then the effective hydrophobic length of the cholesterol molecule may be somewhat less than 20 Å, particularly at higher temperatures. Moreover, although cholesterol induces an "intermediate state" in PC bilayers at temperatures near the phase transition of the pure phospholipid, both X-ray diffraction (Finean, 1990) and ^{2}H-NMR (Sankaram and Thompson, 1990a,b) data indicate that the hydrocarbon thickness and degree of orientational order of the hydrocarbon chains in this intermediate phase is more similar to the gel than to the liquid-crystalline phase of the pure phospholipid, at least at high cholesterol concentrations. Also, evidence has recently been presented that increases in temperature may drive cholesterol from the center toward the polar headgroup region of DPPC bilayers (Reinl *et al.*, 1992). These considerations would all suggest that in fact the optimal degree of hydrophobic mismatch might well occur in a PC with a chain length shorter than 19 carbons. The results presented here, which suggest that 17:0 PC, with a mean hydrophobic chain length of 17.5 Å provides the best match to the cholesterol molecule, are thus entirely reasonable. Moreover, McIntosh et al. (1978) have shown that, at temperatures below the phase transition temperatures of the pure PC's, cholesterol incorporation increases the thickness of 12:0 PC and 16:0 PC bilayers while decreasing the bilayer thickness of 18:0 PC. These observations support our predictions about the differential effect of cholesterol on the gel state of PC's having hydrocarbon chain lengths shorter or longer than 17 carbons.

It is clear from this and other recent studies that the interaction of cholesterol with phospholipid bilayers is a complex process which can be influenced by temperature, cholesterol concentration in the bilayers, and by the structure of the phospholipid molecules themselves. Clearly additional studies, utilizing modern structural, thermodynamic, and spectroscopic techniques, and cholesterol analogues and phospholipid molecules whose polar headgroup and fatty acyl chain structures are systematically varied, will be required to fully understand these interactions. Such studies are currently underway in this laboratory.

Figure II-1. Representative DSC scans of unannealed DPPC bilayers of various cholesterol molar concentrations. DSC scans of DPPC vesicles containing 1,2,3,6,15,25,30, and 40 mol% cholesterol were also performed but are not illustrated here. Endotherms are not adjusted for scan rate or mass of sample.

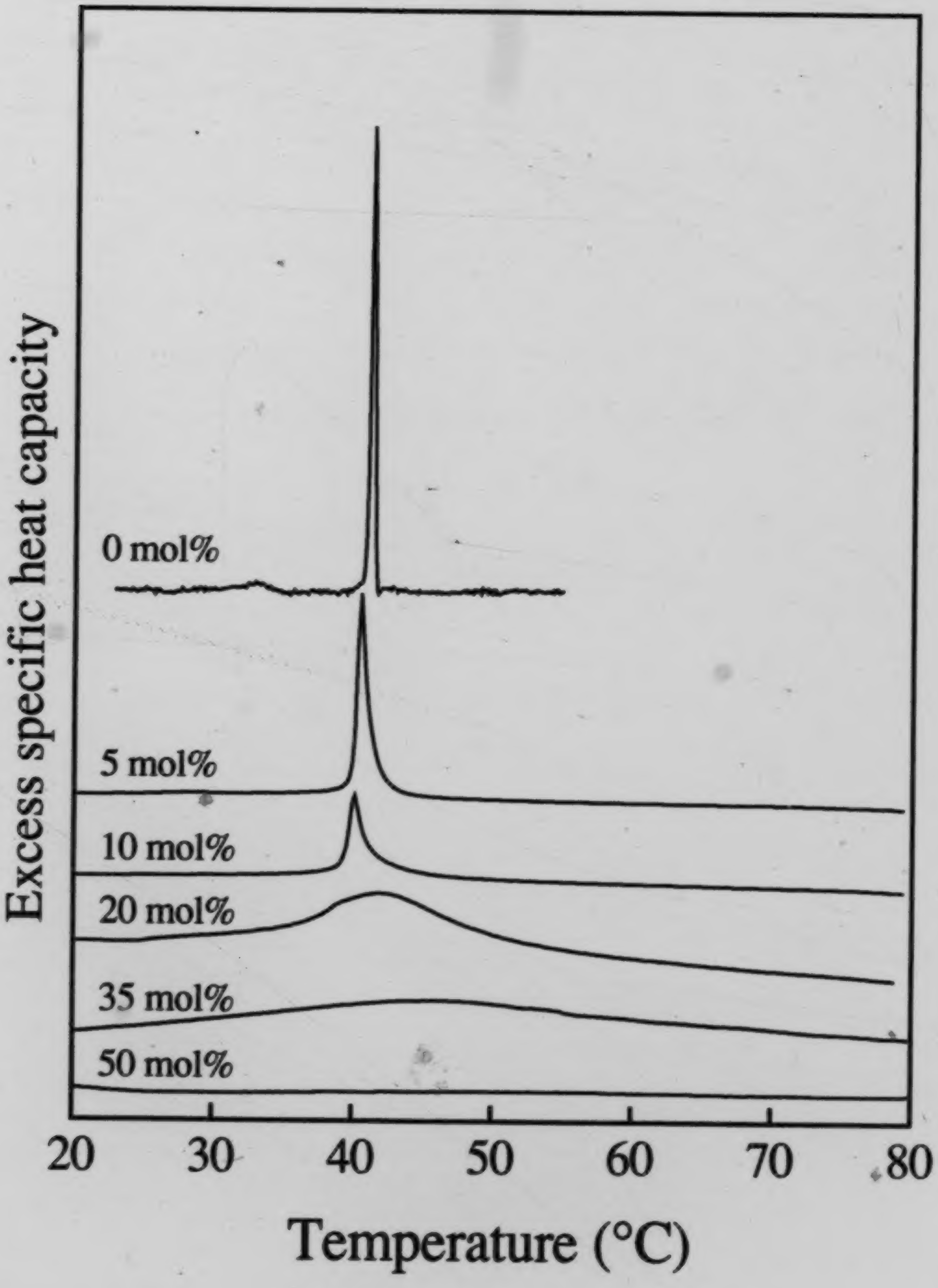

Excess specific heat capacity
0 mol%
5 mol%
10 mol%
20 mol%
35 mol%
50 mol%
20
30
40
50
60
70
80
Temperature (°C)

Figure II-2. Optimized scans of the pretransition (L_{β}'/P_{β}') phase transition of DPPC vesicles containing various molar concentrations of cholesterol. Scans shown are corrected for mass and scan rate to show the relative effect of cholesterol on the pre-transition enthalpy. (1 mol% cholesterol not shown.)

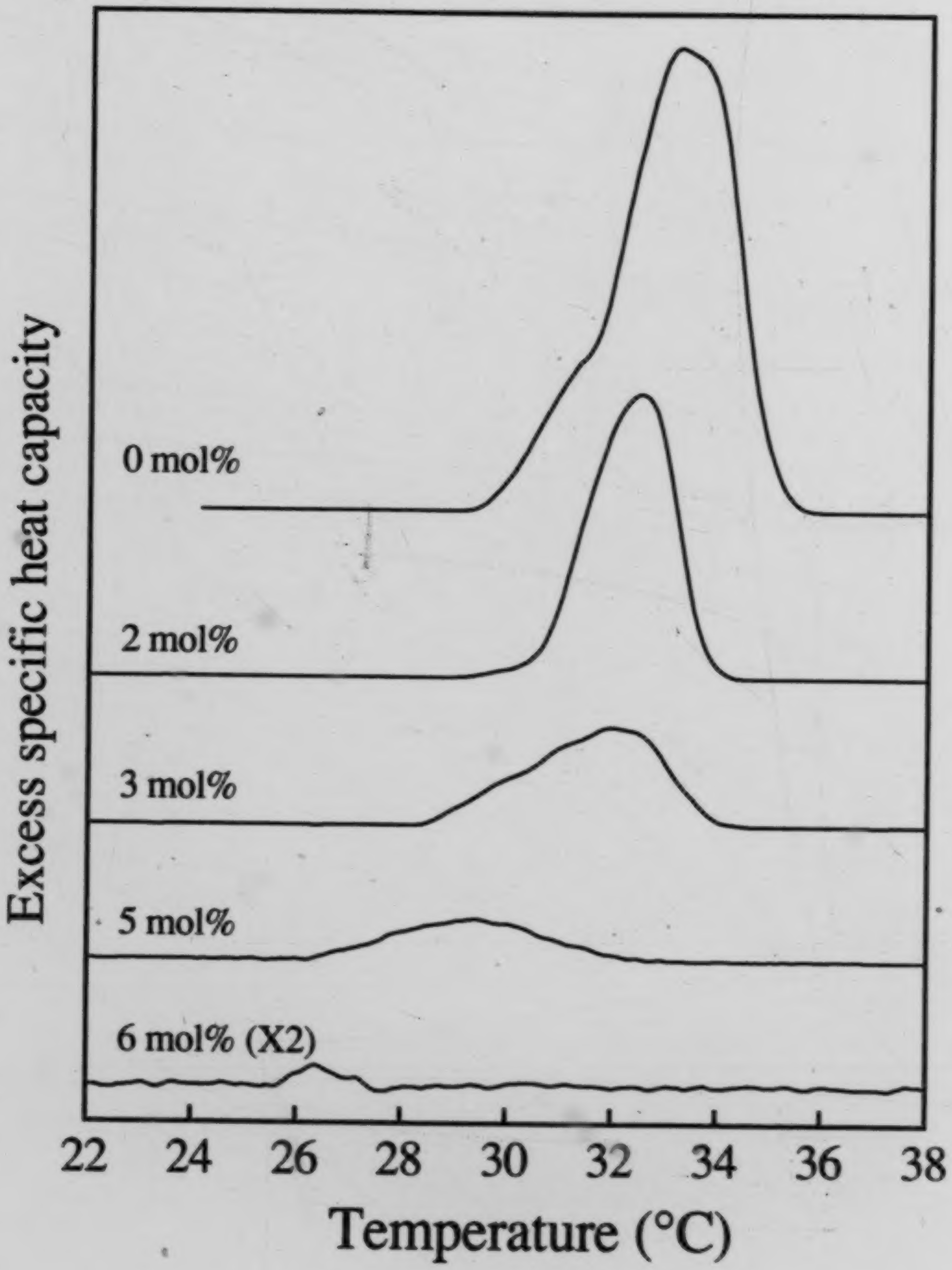

Excess specific heat capacity
0 mol%
2 mol%
3 mol%
5 mol%
6 mol% (X2)
22
24
26
28
30
32
34
36
38
Temperature (°C)

Figure II-3. Sample deconvolution of the DSC endotherm for three concentrations of cholesterol in DPPC vesicles. The lower melting curve represents sharp component and the higher melting curve represents the broad component. Curves are corrected for mass and scan rate.

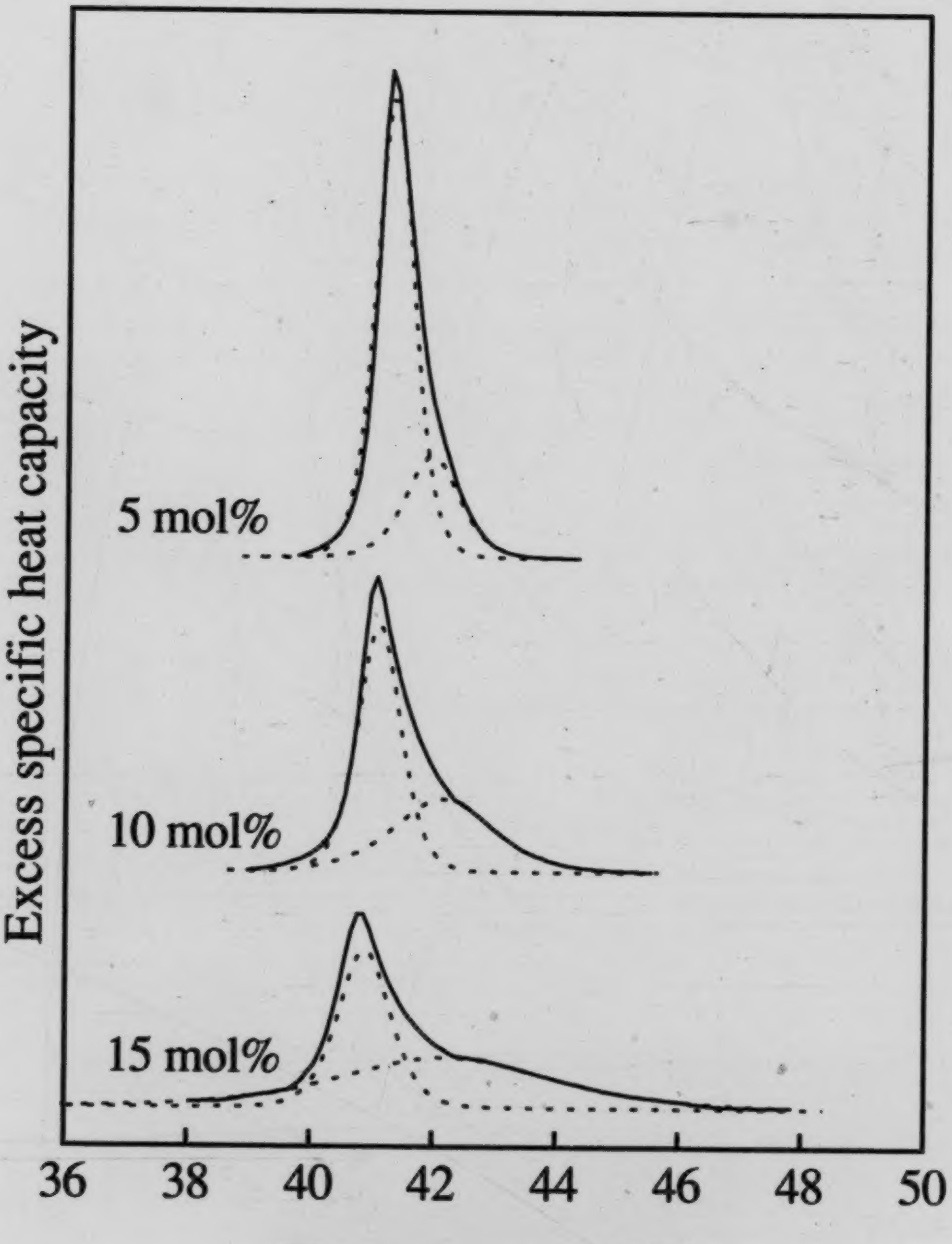

Excess specific heat capacity
5 mol%
10 mol%
15 mol%
36
38
40
42
44
46
48
50
Temperature (°C)

Figure II-4. Representative plot of the net decrease in the temperature of the sharp component of the DSC endotherm as a function of cholesterol concentration. Represented PC's are 14:0 Δ, 16:0 □, 18:0 ▼, and 20:0 ●. Odd chains (13:0, 15:0, 17:0, 19:0, 21:0 PC) are not plotted but are very similar to the even chain PC's plotted above. Transition temperatures corrected for scan rate.

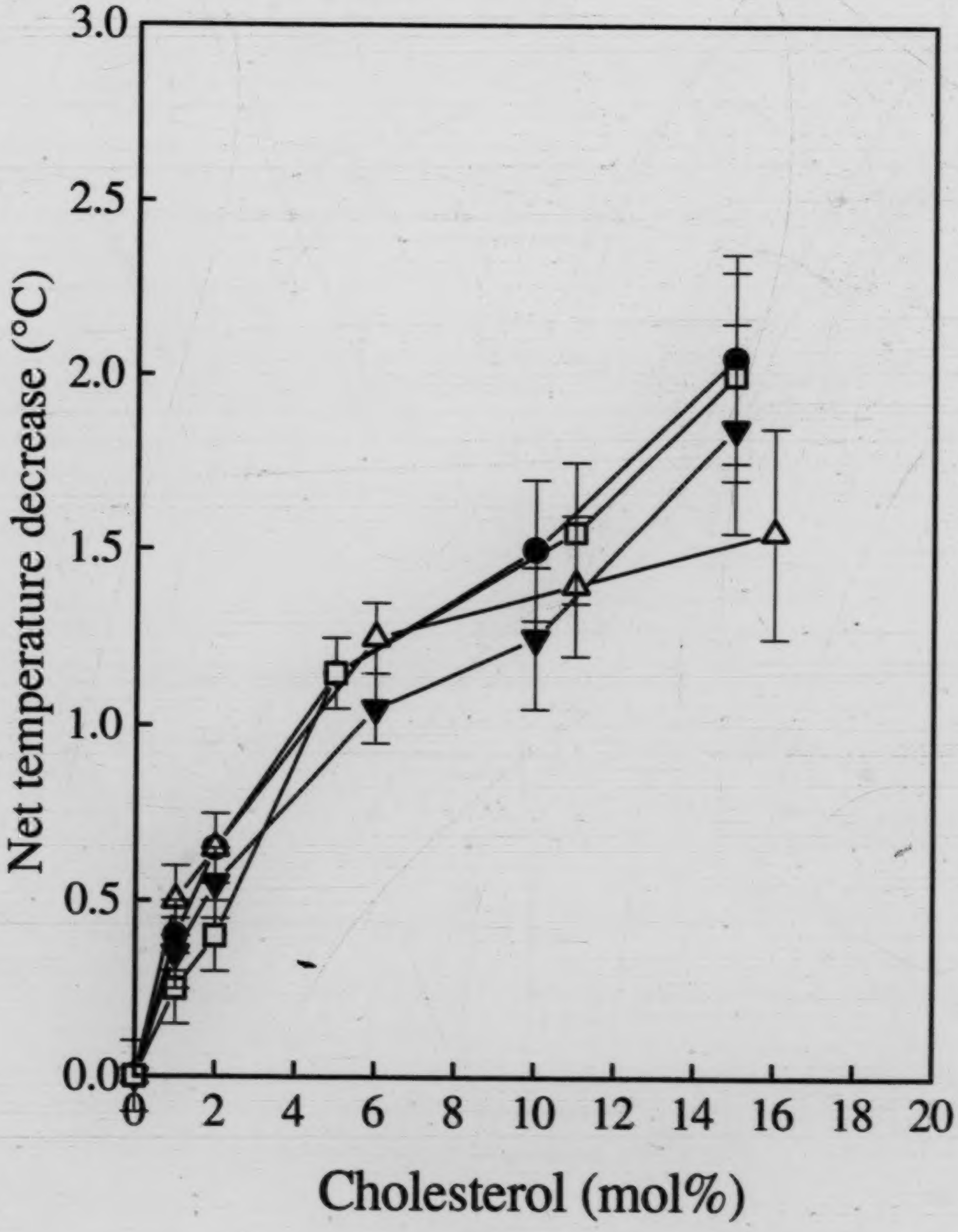

Net temperature decrease (°C)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
18
20
Cholesterol (mol%)

Figure II-5. Representative plots of the enthalpy of the sharp component of the DSC endotherm as a function of cholesterol concentration for the PC homologous series. Represented PC's are 14:0 Δ,16:0 ❑, 18:0 ▼, and 20:0 ●. Data was obtained from the deconvolution of overall endotherms for each chain length (see Fig.3) and averaged. Chain lengths not plotted (13:0, 15:0, 17:0, 19:0, and 21:0 PC) show the same trends as those illustrated in the figure.

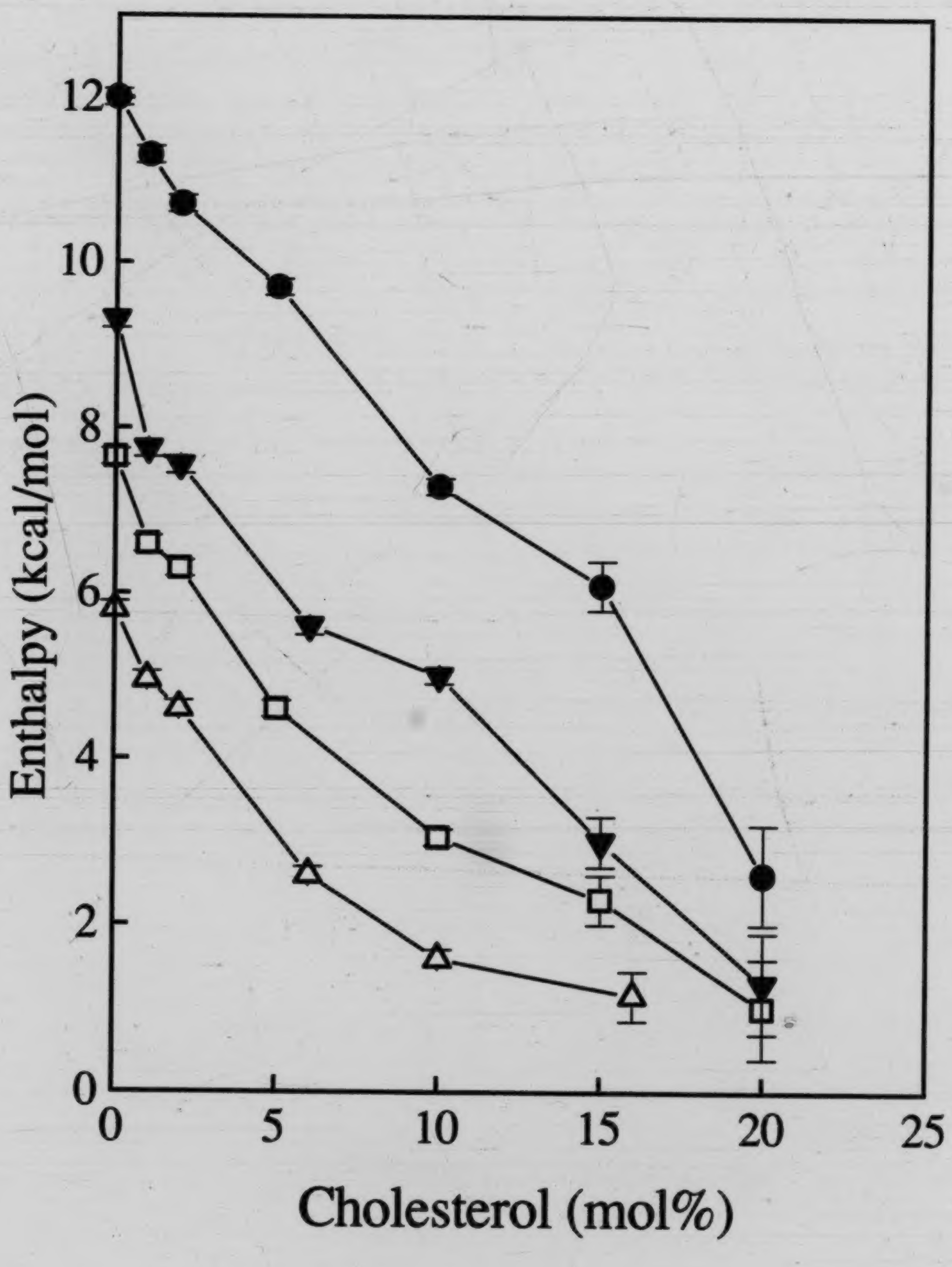

Enthalpy (kcal/mol)
0
2
4
6
8
10
12
Cholesterol (mol%)
0
5
10
15
20
25

Figure II-6. Representative plots of the enthalpy of the broad component of the DSC endotherm as a function of cholesterol concentration for the PC homologous series. Represented PC's are 14:0 Δ, 16:0 ☐, 18:0 ▼, and 20:0 ●. Data was obtained from deconvolution of the overall endotherms for each chain length (see Figure II-3) and averaged. The chain lengths not plotted (13:0, 15:0, 17:0, 19:0,and 21:0 PC) again show the same trends as those shown above.

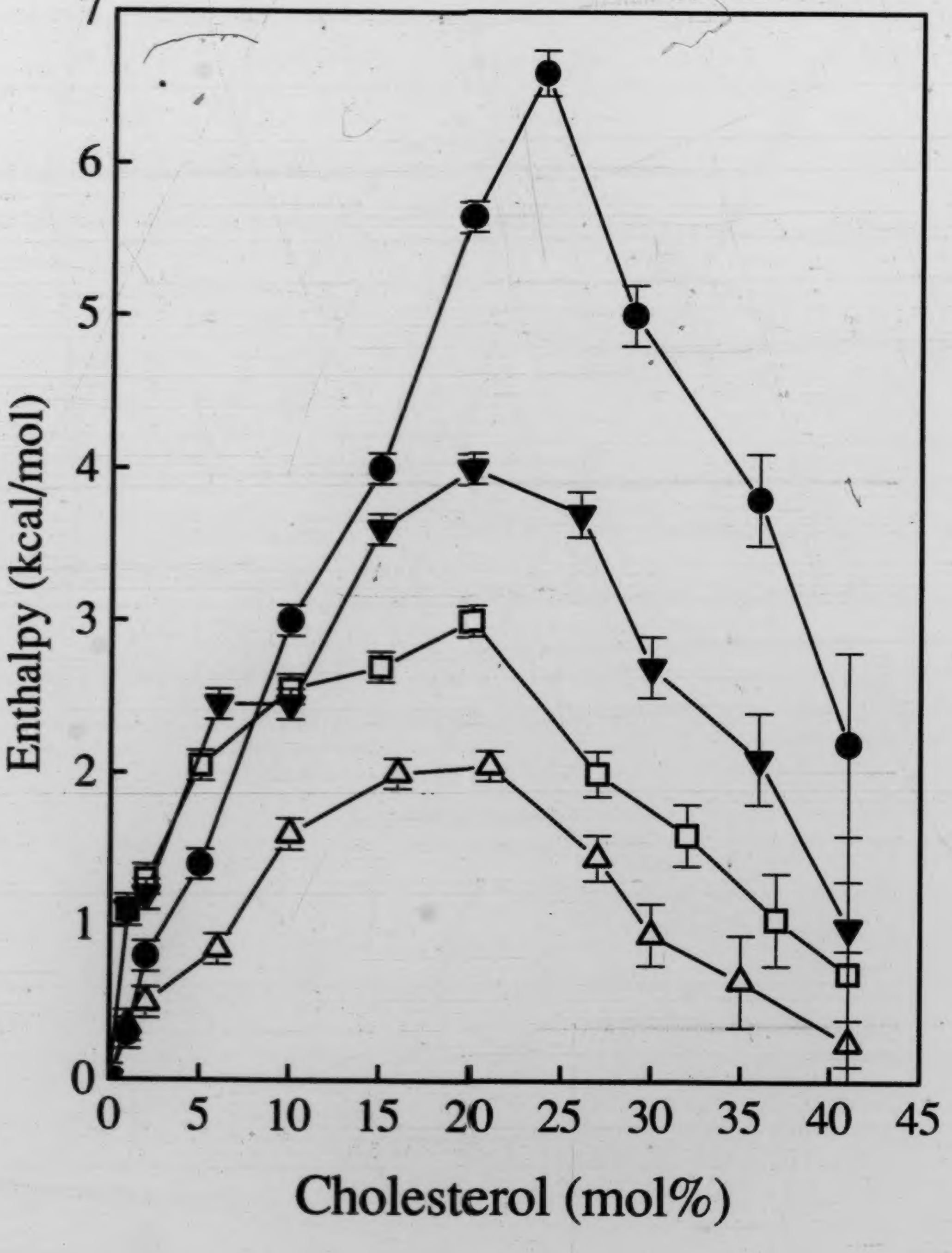

Enthalpy (kcal/mol)
Cholesterol (mol%)
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35
40
45

Figure II-7. Representative plots of the overall main phase transition enthalpies of PC's as a function of cholesterol concentration (PC's shown are 14:0 △, 16:0 □, 18:0 ▼, 20:0 ●). Each point represents the average of at least 3 independent runs. Odd chain PC's, as in Figures II-4 and II-5, are not plotted but exhibit the same trends as the even chain PC's.

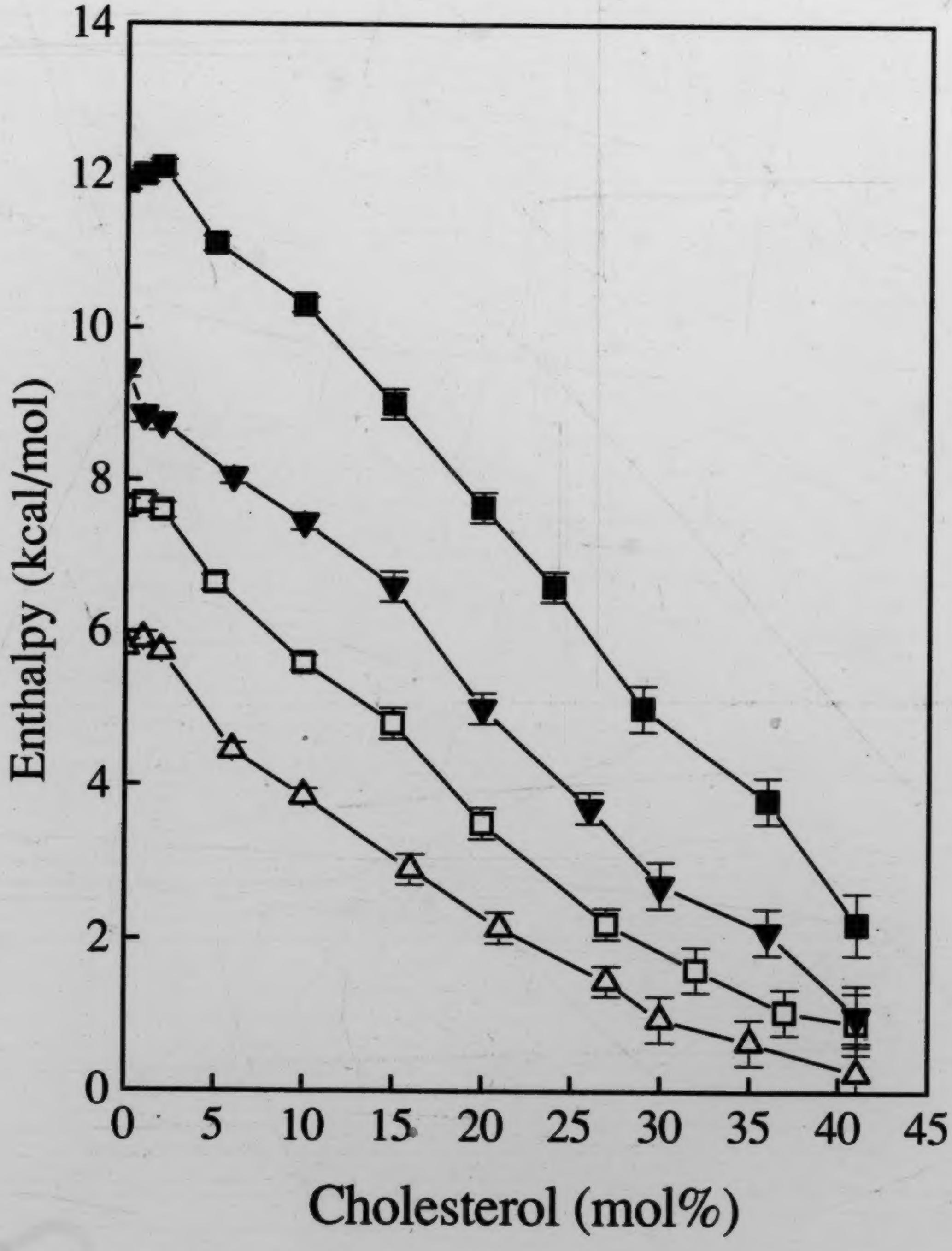

Enthalpy (kcal/mol)
Cholesterol (mol%)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30
35
40
45

Figure II-8. The net transition temperature shift for each PC broad component is shown as a function of cholesterol concentration. Legend for PC's shown is as follows 13:0 ◆, 14:0 ▲, 15:0 ■, 16:0 ▼, 17:0 ●, 18:0 △, 19:0 ◇, 20:0 ❐, 21:0 ▽. Each point is the average of at least 3 runs and all chain lengths are compared to their respective pure chain-melting phase transition temperatures. All transition temperatures are scan-rate corrected.

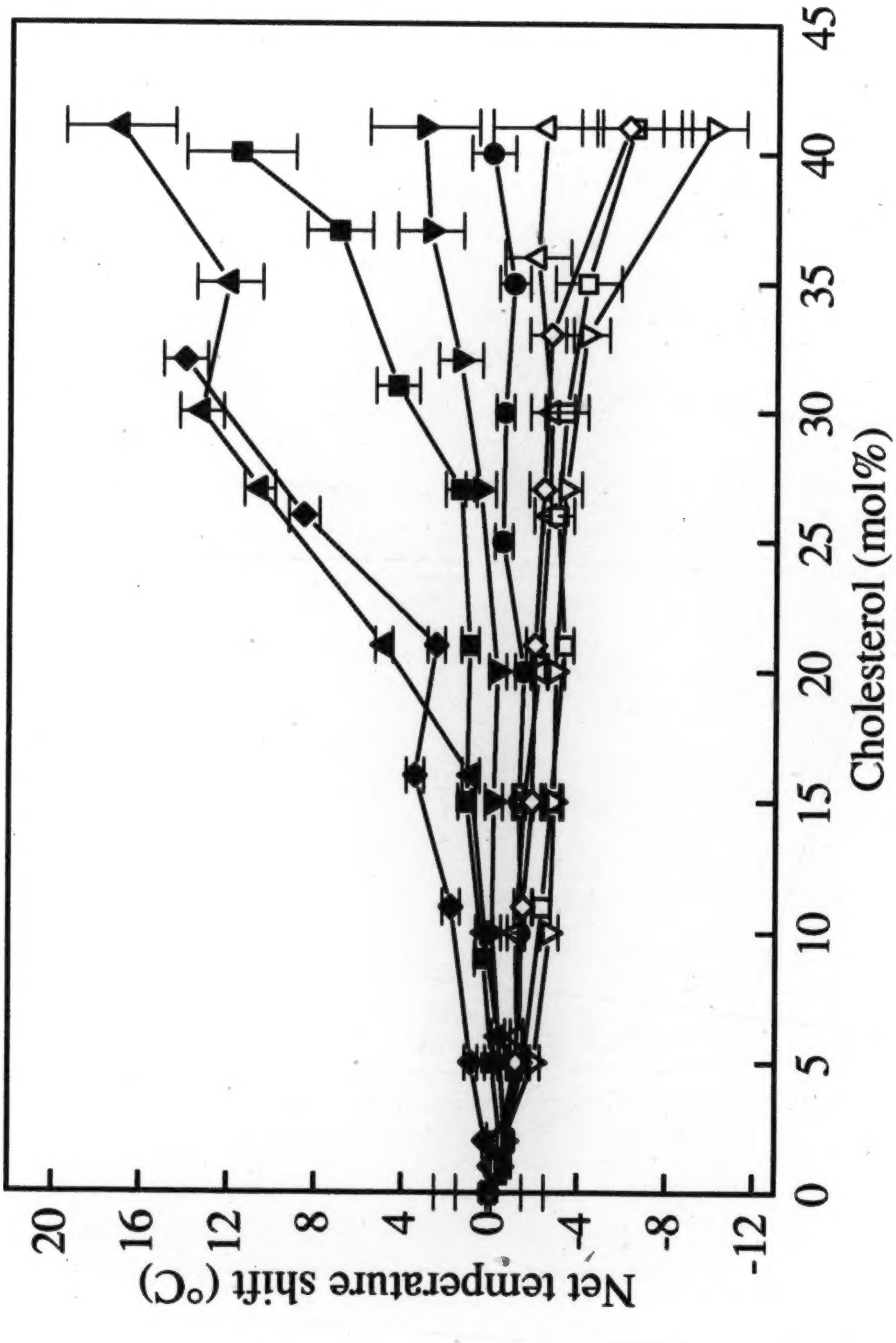
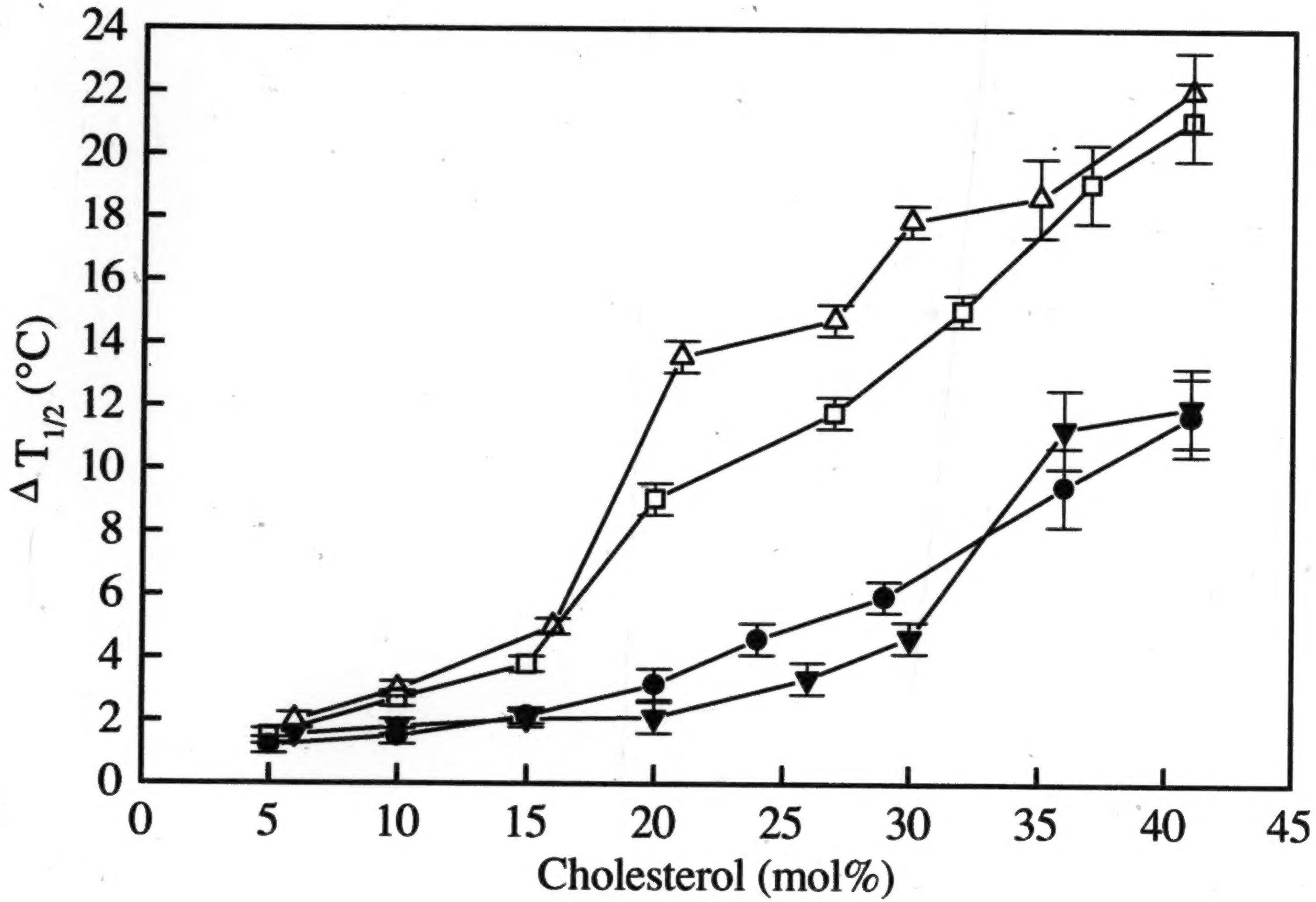

Net temperature shift (°C)
20
16
12
8
4
0
-4
-8
-12
Cholesterol (mol%)
0
5
10
15
20
25
30
35
40
45

Figure II-9. Representative plots of the $\Delta T_{1/2}$ of the broad component of the main phase transition as a function of cholesterol concentration. Legend for PC's in figure are 14:0 Δ, 16:0 ❒, 18:0 ▼, and 20:0 ●. Odd chain PC's were not plotted but the $\Delta T_{1/2}$ values of the 13:0,15:0 and 17:0 PC's were found to behave similarly to the 14:0 and 16:0 PC's, while 19:0 and 21:0 PC's behaved much like the 18:0 and 20:0 PC's.


Δ $T_{1/2}$ (°C)
0
2
4
6
8
10
12
14
16
18
20
22
24
Cholesterol (mol%)
0
5
10
15
20
25
30
35
40
45

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CHAPTER III. NEW ASPECTS OF THE INTERACTION OF CHOLESTEROL WITH DIPALMITOYLPHOSPHATIDYLCHOLINE BILAYERS AS REVEALED BY HIGH-SENSITIVITY DIFFERENTIAL SCANNING CALORIMETRY[3]

INTRODUCTION

Cholesterol, or a closely related sterol, is an essential component of most eukaryotic cellular membranes (Nes and McKean, 1977). Consequently there have been many investigations into the interactions of cholesterol with phospholipid monolayers and bilayers employing a wide variety of physical techniques (for reviews Demel and de Kruijff, 1976; Razin and Rottem, 1978; Yeagle, 1985, 1988). Although we currently know a considerable amount about the manner in which cholesterol alters the organization and mechanical properties of phospholipid bilayers, our understanding of the molecular basis of cholesterol/phospholipid interactions remains incomplete.

Differential scanning calorimetry is a sensitive and non-perturbing thermodynamic technique which has been extensively used to study lipid thermotropic phase transitions in model and biological membranes (Ladbrooke *et al.*, 1968; Hinz and Sturtevant, 1972; Mabrey and Sturtevant, 1976; McElhaney, 1982, 1984; Lewis and McElhaney, 1992). The application of high-sensitivity DSC to cholesterol/DMPC or cholesterol/DPPC binary mixtures has revealed the complex effects of cholesterol incorporation on phospholipid thermotropic phase behavior (Estep *et al.*, 1978; Mabrey *et al.*, 1978; Genz *et al.*, 1986; McMullen *et al.*, 1993; Huang *et al.*, 1993). The incorporation of small amounts of cholesterol progressively decreases the temperature and enthalpy of the pretransition and abolishes it entirely at cholesterol concentrations above 5 mol%. At cholesterol concentrations below 20-25 mol%, the main or chain-melting phase transition endotherm consists of a superimposed sharp and broad component, the former due to the melting of cholesterol-poor and the latter to the melting of cholesterol-rich phospholipid domains. The temperature and cooperativity of the sharp component are reduced slightly with increasing cholesterol incorporation while the enthalpy is reduced markedly and becomes zero at 20-25 mol% cholesterol. In contrast, the broad component increases in temperature and enthalpy but decreases markedly in cooperativity over the same range of cholesterol concentrations. At cholesterol concentrations above 20-25 mol%, the sharp component is abolished and the broad component continues to increase in temperature but to decrease in enthalpy and cooperativity until it is virtually eliminated at about 50 mol% cholesterol (see also Huang *et al.*, 1993). We have recently shown that a qualitatively similar behavior is manifested by the entire homologous series of linear *n*-saturated PCs (McMullen *et al.*, 1993). McMullen *et al.* (1993) have also shown that in addition to the hydrocarbon chain length-independent effects discussed above, cholesterol incorporation has hydrocarbon chain length-dependent effects on the main phase transition of PC bilayers. Specifically, cholesterol incorporation progressively increases the phase transition temperature of the broad component of the DSC endotherm in PCs having hydrocarbon chain lengths of 16 or fewer carbon atoms while progressively decreasing the phase transition temperature in PCs having hydrocarbon chains of 18 or more carbon atoms. This behavior is attributed to the effects of hydrophobic mismatch between the cholesterol molecule and the hydrophobic core of the host PC bilayer (see Mouritsen and Bloom, 1984).

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[3] A version of this chapter has been published. McMullen, T.P.W. and McElhaney, R.N. (1995) *Biochim. Biophys. Acta* 1234: 90-98.

There have been a number of attempts to study the phase equilibria of two-component cholesterol/PC systems and to construct partial phase diagrams (Ladbrooke *et al.*, 1968; Hinz and Sturtevant, 1972; Huang *et al.*, 1993; Shimshick and McConnell, 1973; Gershfeld, 1978; Lentz *et al.*, 1980; Rand et al., 1980; Recktenwald and McConnell, 1981; Blume and Griffin, 1982; Mortensen *et al.*, 1988; Sankaram and Thompson, 1990; Almeida *et al.*, 1992; Thewalt and Bloom, 1992; Linseisen *et al.*, 1993). One notable recent attempt is that of Vist and Davis (1990), who employed DSC and ^{2}H-NMR spectroscopy to determine the phase boundaries of mixtures of cholesterol and chain-perdeuterated DPPC at cholesterol concentrations of 0-25 mol% (see Figure III-1). These workers identified three distinct phases: the liquid-crystalline or L_α phase, the gel or L_β phase, and the β phase. The former phases are pure phospholipid phases while the latter phase contains both phospholipid and cholesterol. The liquid-crystalline phase is characterized by flexible, disordered DPPC hydrocarbon chains undergoing rapid axially symmetric reorientation while the gel phase is characterized by rigid, ordered hydrocarbon chains whose motions are no longer axially symmetric on the ^{2}H-NMR spectroscopic timescale. The cholesterol-rich β phase is characterized by relatively ordered, rigid hydrocarbon chains but rapid axially symmetric reorientation. In addition, these workers identified three regions of two-phase coexistence. The first is a narrow L_α/L_β phase coexistence region between 0 and about 7 mol% cholesterol occurring just below the chain-melting temperature of the pure phospholipid. The second boundary occurs between 7.5 and 22 mol% cholesterol and runs from 37 °C down to at least 30 °C; within this region the gel and β phases coexist. The third two-phase region lies above 37 °C, beginning at a eutectic point between 7.5 and 10 mol% cholesterol and ending at about 20 mol% cholesterol; in this region the L_α and β phases are in equilibrium. As well, a three-phase line at 37 °C extends from the eutectic point up to at least 20 mol% cholesterol. The phase diagram constructed by Vist and Davis (1990) is widely quoted and has received both experimental and theoretical support (Thewalt and Bloom, 1992; Linseisen *et al.*, 1993; Ipsen *et al.*, 1987). Moreover, this same diagram is thought to describe the phase behavior of other cholesterol/PC systems varying in PC hydrocarbon chain length and unsaturation.

The existence of a eutectic or triple point near 7.5 mol% cholesterol was deduced by Vist and Davis (1990) from an apparent sharpening of the DSC endotherm corresponding to the main phase transition of chain-perdeuterated DPPC. However, in our previous study of DPPC/cholesterol mixtures, we (McMullen *et al.*, 1993) and others (Mabrey and Sturtevant, 1976; Estep *et al.*, 1978; Mabrey *et al.*, 1978; Genz *et al.*, 1986) did not detect any discontinuity in the behavior of the sharp and broad components of the DSC endotherm between 0 and 20-25 mol% cholesterol. To clarify this point, we have carefully reinvestigated the thermotropic phase behavior of both annealed and unannealed DPPC/cholesterol mixtures containing 0 to 20 mol% cholesterol using very low scan rates and 1 mol% intervals over the cholesterol concentration range of the presumed eutectic point. Although the apparent cooperativity of the overall DSC endotherm, measured as $\Delta T_{1/2}$, does appear to reach a local minimum at approximately 7 mol% cholesterol, this behavior is actually an artifact due to characteristic alterations in the temperature, enthalpy, and cooperativity of the sharp and broad components of the DSC endotherm as cholesterol concentrations in the bilayer progressively increase. We confirm that this is the case by comparative studies of cholesterol interactions with DMPC, DSPC, and DAPC bilayers. We also point out other features of our DSC data which are not in accord with the phase diagram constructed by Vist and Davis (1990) and describe the effects of cholesterol incorporation of the various gel phases of DPPC. Based on our DSC results as well as recent spectroscopic studies (Huang *et al.*, 1993; Reinl *et al.*, 1992), we then propose a modified cholesterol/DPPC temperature-composition diagram and indicate how changes in PC chain length alter the temperature/composition plot. Finally, we interpret our results for the cholesterol/CnPC phase equilibria in light of recent models for the maintenance of the non-uniform distribution of cholesterol within eukaryotic cells (Bretscher and Munro, 1993) and non-protein-mediated PC/cholesterol vesicular budding (Julicher and Lipowsky, 1993; Dobereiner *et al.*, 1993).

MATERIALS AND METHODS

The PCs used in this experiment were synthesized and purified by the methods used by Lewis and McElhaney (1985), which have been shown to yield highly pure samples. The cholesterol was purchased from Fisher Chemicals (Fairlawn, NJ) and recrystallized twice from ethanol before use. PC/cholesterol mixtures were prepared from stock solutions of chloroform and dried under N_2 and evaporated to dryness under vacuum overnight. The dispersions were hydrated with milli-Q water with repeated vortexing and heating to approximately 20 °C above the bilayer main phase transition. No significant differences were observed in the thermotropic behavior of these mixtures when different combinations of chloroform and methanol stock solvents were used to prepared the mixtures or different buffers were used to hydrate the bilayers. The calorimetry was done on a Hart Scientific high-sensitivity differential scanning calorimeter (Pleasant Grove, UT). For all samples a scan rate of 5 °C/hr was used. This slow scan rate ensures that there is a minimum of endotherm curve distortion due to instrumental time lag. Sample runs were repeated at least three times to ensure reproducibility. We also analyzed the thermotropic phase behavior of these same samples using a Microcal MC-2 calorimeter with a scan rate of 11.0 °C/hr. The results were essentially identical to that obtained with the Hart calorimeter. Decomposition of the multicomponent melting curves was done using Microcal's Origin and DA-2 software. Briefly, this computer program approximates the multicomponent melting thermograms as a linear combination of multiple, independent, two-state transitions. The curve broadening is expressed in terms of the van't Hoff enthalpy, which is evaluated by the equation $\Delta H_{vH}=4RT_m{}^2(c_{max}/\Delta q)$, where c_{max} is the excess specific heat capacity and Δq is the area under the curve. This protocol accurately reproduces the experimental DSC endotherms and has been used in previous studies (see also Estep *et al.*, 1978; Mabrey *et al.*, 1978; McMullen *et al.*, 1993; Huang *et al.*, 1993). Although other methods of estimating the temperature, enthalpy and cooperativity of the components of these DSC endotherms could be employed, these would yield qualitatively similar results. In addition to our curve decomposition protocol, we analyzed the differences between pure DPPC and DPPC/cholesterol (2-5 mol%) endotherms using a simple thermogram subtraction routine. This procedure involves the subtraction of a pure DPPC endotherm from the endotherms of cholesterol/DPPC mixtures containing the same amount of DPPC scanned at the same scan rate and matched precisely for T_m. This routine allows us to directly observe qualitative differences in DPPC curve shape upon cholesterol incorporation.

RESULTS

Some representative high-sensitivity DSC plots of low-temperature annealed, aqueous, multilamellar dispersions of DPPC containing 0 to 20 mol% cholesterol are shown in Figure III-2. In the absence of cholesterol, annealed DPPC bilayers exhibit three endotherms upon heating, a subtransition at approximately 18 °C, a pretransition at 34 °C, and the main transition at 41.4 °C. The subtransition arises from the conversion of a crystalline gel (L_c') phase to the lamellar gel (L_{β}') phase, the pretransition from the conversion of the L_{β}' phase to the rippled gel (P_{β}') phase, and the main transition from a conversion of the P_{β}' phase to the lamellar liquid-crystalline (L_{α}) phase. The enthalpy of the subtransition decreases progressively with increases in cholesterol content with little change in the transition temperature and is abolished completely at 20 mol% cholesterol. FTIR analysis of the CH_2 bending band of cholesterol-containing DPPC bilayers indicates that the L_c' phase observed in these mixtures is structurally identical to that observed in pure DPPC bilayers (Lewis and McElhaney, 1990) (data not presented). For the pretransition, both the enthalpy and the temperature of the transition decrease in a linear manner with increasing cholesterol concentration and the pretransition completely disappears above 5 mol% cholesterol, as shown previously (McMullen *et al.*, 1993). The enthalpy of the overall main phase transition decreases with increases in cholesterol concentration (see Figure III-4a) while the transition temperature decreases slightly (see Figure III-4b). Interestingly, the apparent cooperativity of the overall chain-melting phase transition (inversely related to the $\Delta T_{1/2}$ value), appears to decrease from 0 to 3-4 mol%, to increase from 4-7 mol%, and then to decrease again at cholesterol concentrations above 7 mol% (see Figure III-4c). This result would appear to confirm the observations of Vist and Davis (1990) and to support the existence of a eutectic point near 7 mol% cholesterol. Note, however, that the cooperativity of the overall main phase transition at 7 mol% cholesterol is considerably less than that of pure DPPC. The effects of cholesterol on the behavior of the pretransition and main transition of unannealed cholesterol/DPPC mixtures is exactly the same as for the corresponding annealed samples as described above (data not presented).

We (McMullen *et al.*, 1993) and others (Estep *et al.*, 1978; Mabrey *et al.*, 1978; Huang *et al.*, 1993) have established that the asymmetric DSC endotherm corresponding to the main phase transition of DPPC (and other linear saturated PC's) consists of superimposed sharp and broad melting components at cholesterol concentrations of approximately 1-2 mol% to 20-25 mol%. Moreover, the sharp component has been assigned to the chain-melting phase transition of cholesterol-poor PC domains and the broad component to the melting of cholesterol-rich PC domains. Accordingly, we have decomposed the DSC endotherms presented in Figure III-2 into sharp and broad components and the results of the decomposition are presented in Figure III-3. As illustrated in Figures III-4a,b,c the sharp component enthalpy decreases substantially, the phase transition temperature decreases slightly (about 1.5 °C), and the cooperativity decreases moderately with increases in cholesterol concentration from 0 to 15 mol%. In contrast, the broad component enthalpy increases markedly, the phase transition temperature increases slightly (about 2.0-2.5 °C), and the cooperativity decreases to a greater extent than for the sharp component. Clearly, each of the above thermodynamic parameters for both the sharp and broad phase transitions changes monotonically with increases in the level of cholesterol incorporation over the range 1-2 to 20 mol%. Thus there is no evidence for a eutectic point or any discontinuity in phase behavior near 7.5 mol% cholesterol. In addition, our results clearly show that the broad melting component is observed at temperatures both above and below the T_m of pure lipid domains, as illustrated in Figure III-3 for DPPC.

To ensure that the decomposition procedure of the cholesterol/DPPC endotherms is an accurate description of the changes in the overall endotherm curve shape with increasing cholesterol, we also used a thermogram-subtraction technique to identify changes in endotherm contour. This method does not assume a two-state transition, nor does it apply curve-fitting routines to analyze the curve profile, it simply demonstrates the differences between the curves matched for T_m. This routine is based on the fact that the $\Delta T_{1/2}$ value of the pure lipid endotherm is very sensitive to the addition of an added "impurity" and that changes in curve shape are indicative of the nature of the interaction of the impurity with the lipid bilayer (McElhaney, 1982; Biltonen and Lichtenberg 1993). Shown in Figure III-5 is an example of the results for the subtraction of a pure DPPC endotherm from endotherms with the same quantity of DPPC but containing 2 or 5 mol% cholesterol. In accord with our decomposition results, cholesterol-induced differences in the shape as well as in the cooperativity of the DPPC endotherms are clearly observed even at these low cholesterol concentrations. Note that at the lower cholesterol concentration (2 mol%), the DPPC endotherm is broadened primarily on the low-temperature side while at the higher cholesterol concentration (5 mol%), this endotherm is broadened primarily on the high-temperature side. This asymmetric broadening of the endotherm induced by these low concentrations of cholesterol supports the existence of two components in the DSC chain-melting transition whose peak temperature change in a differential fashion with increasing cholesterol concentration. These results contrast with those of Vist and Davis (1990), who reported symmetric, one-component DSC endotherms for DPPC/cholesterol mixtures containing less than 7.5 mol% cholesterol.

We have carried out a parallel series of high-sensitivity DSC experiments in which the effect of low concentrations of cholesterol on the thermotropic phase behavior of DMPC, DSPC, and DAPC bilayers were also investigated so that the effect of variations in PC hydrocarbon chain could be ascertained. The original and decomposed DSC thermograms for a series of progressively increasing cholesterol concentrations in bilayers of these three PC's are presented in Figure III-6. In all cases the T_m of the sharp component of the DSC endotherms decreases slightly as the concentration of cholesterol incorporated into the PC bilayer increases. However, the T_m of the broader component shifts upward relative to that of the sharp component for DMPC and for DPPC, while the opposite is the case for DSPC and DAPC. In all cases the T_m of the broad component at very low cholesterol concentrations is slightly less than that of the sharp component, such that crossing over of the T_m's will be observed for DMPC and DPPC but not for DSPC and DAPC. Moreover, since the upward shift of the T_m of the broad component of DMPC is more pronounced than that of DPPC at comparable cholesterol concentrations, one would predict that the crossing over of the T_m's of the sharp and broad components would occur at lower cholesterol concentrations in DMPC than in DPPC, and this indeed is observed. That this phenomenon of T_m crossover is in turn related to the apparent decrease in the overall $\Delta T_{1/2}$ of the overall DSC endotherm at modest cholesterol concentrations is amply illustrated in Figure III-7. These results clearly establish that the local minimum in $\Delta T_{1/2}$ values at cholesterol concentrations of 5-7 mol% is observed only with the shorter chain PC's, and that this local minimum is the result of a superposition of the sharp and broad components of the DSC endotherm.

DISCUSSION

Our investigation of the thermotropic phase behavior of cholesterol/DPPC mixtures clearly shows that the sharpening of the DSC endotherm observed by Vist and Davis (1990) arises because of the divergent effects of cholesterol on the various thermodynamic parameters of the sharp and broad components of the main phase transition. At low cholesterol concentrations (1 to 3 mol%), the cooperativity of the unresolved endotherm decreases because the cooperativity of both the sharp and broad components are decreasing. However, because the phase transition temperature of the broad component is initially slightly lower (about 1 °C) than the sharp component but the two components are converging in temperature with increasing cholesterol concentration, the initial decrease in the cooperativity of the unresolved endotherm is somewhat attenuated. Moreover, as the transition temperature of the sharp component continues to decrease and that of the broad component to increase, these component transitions superimpose on the temperature scale. This results in a slight sharpening of the unresolved endotherm in the cholesterol concentration range of 3 to 7 mol%, despite the fact that the cooperativity of both the sharp and broad components continue to decrease. At higher cholesterol concentrations the increasing divergence of the sharp and broad components on the temperature scale, as well as the increasing dominant contribution of the broad component, again cause the cooperativity of the unresolved endotherm to decrease, now quite rapidly. We confirmed this interpretation of our results by studies of longer chain length cholesterol/PC mixtures (DSPC and DAPC), in which the sharp and broad component T_m values do not cross at all, resulting in no apparent increase in endotherm cooperativity at any cholesterol concentration. This explains why other investigators have reported that the multicomponent melting of PC/cholesterol mixtures is less obvious in longer chain PC's (McMullen *et al.*, 1993; Davis and Keough, 1983, 1984). We stress that the cooperativity of both the sharp and broad components decrease continuously with increases in cholesterol concentration, which is incompatible with the existence of a eutectic point near 7.5 mol% cholesterol for any cholesterol/CnPC mixture.

Another aspect of the present and previous (Estep *et al.*, 1978; Mabrey *et al.*, 1978; Genz *et al.*, 1986; McMullen *et al.*, 1993; Huang *et al.*, 1993) DSC studies of cholesterol/PC mixtures which does not appear to be compatible with the phase diagram proposed by Vist and Davis (1990) relates to the cholesterol concentration at which the cholesterol-rich β–phase first arises. Vist and Davis (1990) report that the β phase exists only at concentrations above the proposed eutectic point at approximately 7.5-10 mol% cholesterol. This conclusion arises from the asymmetric shape of their DSC endotherms, which appeared to show that the broad component exists exclusively as a higher temperature shoulder on the sharp component. However, the asymmetric endotherms for DPPC/cholesterol mixtures which we observe over the cholesterol concentration range 2 to 20 mol% are very well fit by a superposition of a single sharp and a single broad component, even at very low cholesterol concentrations. Moreover, the limits of the broad component clearly extend to temperatures below as well as above those of the sharp component. We also note that Huang *et al.* (1993) indicate that their ^{2}H-NMR spectra did not exhibit clearly separable components which could be attributed to the gel or L_α phase, nor did they observe a characteristic sharpening of the ^{2}H-NMR resonances that Vist and Davis (1990) reported. Huang *et al.* (1993) also state that ^{13}C-NMR spectroscopic data is crucial to correctly interpreting the ^{2}H-NMR spectral data. Thus the rationale used by Vist and Davis (1990) in support of the existence of the L_α/β two-phase coexistence region only at cholesterol levels greater than 7.5 mol% is not supported by our results or those of Huang *et al.* (1993).

The phase diagram of Vist and Davis (1990) is also incompatible with the present and previous DSC results in its identification of only a single gel phase. In annealed DPPC bilayers without cholesterol, three different gel phases (the L_c', L_β' and P_β') clearly exist. Our DSC data indicates that the L_β'/P_β' phase transition is eventually abolished by 5 mol% cholesterol while the L_c'/L_β' phase transition persists until approximately 20 mol% cholesterol (see also Koynova *et al.*, 1985). The persistence of the L_c' phase up to approximately 20 mol% cholesterol indicates that extended arrays of pure lipid exist and that their size and/or number is inversely proportional to the level of cholesterol incorporation. This is important evidence directly demonstrating the existence of a phase-separated mixture containing pure DPPC and DPPC-cholesterol domains at cholesterol concentrations between 0 and 20 mol%. In addition, the incorporation of cholesterol results in the loss of the rippled structure characteristic of the P_{β}' phase (Matuoka *et al.*, 1994) and of the characteristic chain tilt of all three gel phases of pure DPPC. Thus the L_{β}' and P_{β}' (and possibly also the low-temperature annealed L_{c}') phases of DPPC which exist in the absence of cholesterol are converted to a new, untilted gel phase (the L_{β} phase) upon the incorporation of relatively small amounts of cholesterol (5 mol%) (see Finean, 1990).

Using our DSC data as well as information from more recent PC/cholesterol spectroscopic studies (McMullen *et al.*, 1993; Reinl *et al.*, 1992; Huang *et al.*, 1993) we propose a revised form of the cholesterol/DPPC "phase diagram" (or perhaps more accurately a temperature/composition diagram) in Figure III-8. Similar to the majority of prior cholesterol/PC phase diagrams (Shimshick and McConnell, 1973; Gershfeld, 1978; Lentz *et al.*, 1980; Rubenstein et al., 1980; Rand et al., 1980; Recktenwald and McConnell, 1981; Blume and Griffin, 1982; Mortensen *et al.*, 1988; Sankaram and Thompson, 1990; Vist and Davis, 1990), our temperature/composition diagram can be described in two segments, DPPC with less than 20 mol% cholesterol and DPPC with greater than 20 mol% cholesterol. Below 20 mol% cholesterol the simultaneous existence of pure lipid as well as lipid-sterol domains accounts for the thermotropic behavior of the bilayer, while above 20 mol% cholesterol the pure lipid domains are abolished. This interpretation is based on a lipid/sterol interaction stoichiometry of approximately 3.5 lipid molecules to one cholesterol molecule (Engleman and Rothman, 1972), which is universally accepted for DPPC and has been shown to apply to different chain length PC/cholesterol mixtures by McMullen *et al.* (1993). For cholesterol concentrations of 0 to 20 mol% in annealed DPPC/cholesterol bilayers, the first transition observed with increasing temperature is the subtransition (L_{c}'/L_{β}') which persists up to 18-20 mol% cholesterol. The L_{β}' phase is stable until approximately 33°C, afterwhich the L_{β}' phase converts to the P_{β}' phase, but only at cholesterol concentrations less than 5 mol%. Finally, the P_{β}' and L_{β}' phases convert to the L_{α} phase to a limit of 20 mol% cholesterol. However, unlike Vist and Davis (1990), we do not expand the P_{β}'/L_{α} phase boundaries to show a large phase coexistence region which then proceeds to a eutectic point and a single L_o or β phase at cholesterol concentrations above 7.5 mol%. Instead, we describe two distinct L_o regions, the liquid-crystalline-like $L_{o\alpha}$ and gel-like $L_{o\beta}$ region. Our classification of two different L_o regions is based on the experimentally observable chain-melting transition seen by FTIR and DSC (McMullen *et al.*, 1993; Huang *et al.*, 1993; Reinl *et al.*, 1992; McMullen *et al.*, 1994; Umemura *et al.*, 1980), as well as the ^{13}C-NMR and ^{2}H-NMR spectroscopic data of Reinl *et al.* (1992) and Huang *et al.* (1993), which demonstrate that the L_o or β phase is not homogeneous with respect to temperature. In fact, the ^{2}H-NMR and ^{13}C-NMR data of Huang *et al.* (1993) is in close agreement with the DSC study of McMullen *et al.* (1993) regarding the chain length-dependent transition temperature and boundaries of the melting of cholesterol-rich DPPC and DSPC domains. The differences in $L_{o\alpha}$ and $L_{o\beta}$ thermotropic phase behavior may be manifest structurally as changes in the orientational order of the PC hydrocarbon chains and the cholesterol C17 side chain, as well as the relative position of the cholesterol molecule in the host PC bilayer (McMullen *et al.*, 1993; Sankaram and Thompson, 1990; Reinl *et al.*, 1992; Habercorn *et al.*, 1977; Wu and Chi, 1991). We also indicate that the origin of these regions is at very low cholesterol concentrations and that only at very low, experimentally inaccessible cholesterol concentrations does one observe ideal miscibility and possibly the existence of a eutectic point (Cevc and Marsh, 1987). Within the boundaries of the broad component transition (denoted by ■) but above or below the sharp component phase transition, three different phases apparently coexist; (*^{1}) L_α+$L_{o\alpha}$+$L_{o\beta}$ (above) or (*^{2}) L_β+$L_{o\alpha}$+$L_{o\beta}$ (below). At approximately 20-25 mol% cholesterol only the $L_{o\alpha}$ and $L_{o\beta}$ phases persist and as the level of cholesterol increases beyond 20-25 mol%, so does the temperature range for the coexistence of these two regions. At 50 mol% cholesterol pure cholesterol domains form and a cooperative lipid transition is no longer observable. Moreover, while the stoichiometry of cholesterol/PC interactions does not vary with chain length, it is clear that there is some specificity to cholesterol/PC interactions, primarily due to the phenomena of hydrophobic mismatch (see McMullen *et al.*, 1993). Thus variations in PC hydrocarbon chain length affect both the direction and magnitude of the temperature shift as well as the cooperativity of the broad component DSC endotherm. As a result, the $L_{o\alpha}$ and $L_{o\beta}$ region boundaries of the DPPC/cholesterol temperature/composition plot (denoted ■) will change with changes in PC chain length. This contradicts previous theoretical predictions and ^{2}H-NMR spectroscopic evidence that PC/cholesterol interactions demonstrate no specificity regarding the PC hydrocarbon chain composition (Vist and Davis, 1990; Thewalt and Bloom, 1992; Linseisen *et al.*, 1993; Ipsen *et al.*, 1987). It should also be noted that sterol/phospholipid thermotropic phase behavior can be profoundly altered by subtle structural changes in the sterol molecule or in the polar headgroup and hydrocarbon chains of the host phospholipid bilayer (Demel and de Kruijff, 1976; Yeagle, 1988; McElhaney 1982, 1984; Lewis and McElhaney, 1992; McMullen *et al.*, 1994).

Our DSC results indicate that phase separation of cholesterol-rich and cholesterol-poor domains occurs even at very low cholesterol concentrations, such as exist in various intracellular membranes. Interestingly, this finding has implications regarding the maintenance of a non-uniform distribution of cholesterol in eukaryotic cell membranes and possibly also in the process of protein sorting. Recently, Bretscher and Munro (1993) proposed that the formation of cholesterol-rich phospholipid or sphingolipid domains could serve as a sorting mechanism for membrane proteins destined for the plasma membrane. This process was postulated to occur as a direct consequence of the greater hydrophobic thickness of the more ordered cholesterol/lipid domains and of the longer transmembrane α-helices of the plasma membrane proteins. For such a mechanism to be effective, the phase separation of cholesterol/lipid domains would have to occur at low cholesterol concentrations to allow for selective concentration of cholesterol from the endoplasmic reticulum through the Golgi network towards the plasma membrane. Our work clearly demonstrates that cholesterol-induced phase separation is possible even at very low cholesterol concentrations. In addition, theoretical and experimental studies have shown that phase-separated PC/cholesterol vesicles may undergo dramatic shape fluctuations which can lead to vesicular fission even without the assistance of membrane-associated proteins (Julicher and Lipowsky, 1993; Dobereiner *et al.*, 1993). This budding process was related to the formation of liquid-ordered ($L_{o\alpha}$ in our phase diagram) and liquid-disordered (L_α in our phase diagram) DMPC/cholesterol phase-separated regions (Dobereiner *et al.*, 1993). We believe that cholesterol may in fact be required for the domain-induced budding process due to its ability to form phase-separated liquid-ordered domains even in the heterogeneous and fluid environment of biological membranes (Julicher and Lipowsky, 1993). Thus changes in bilayer sterol/lipid composition via phase separation may be selectively driving the vesicular transport of the relatively thicker cholesterol-enriched domains and their associated proteins. Although proteins may also induce or facilitate vesicular budding in biological membranes, the physical properties of the bilayer may still be crucial for this process *in vivo*. Clearly much work remains to be done to understand the complex interactions of cholesterol with the great diversity of the lipids found in natural membranes.

Figure III-1. The DPPC/cholesterol phase diagram proposed by Vist and Davis (1990). Used with permission.

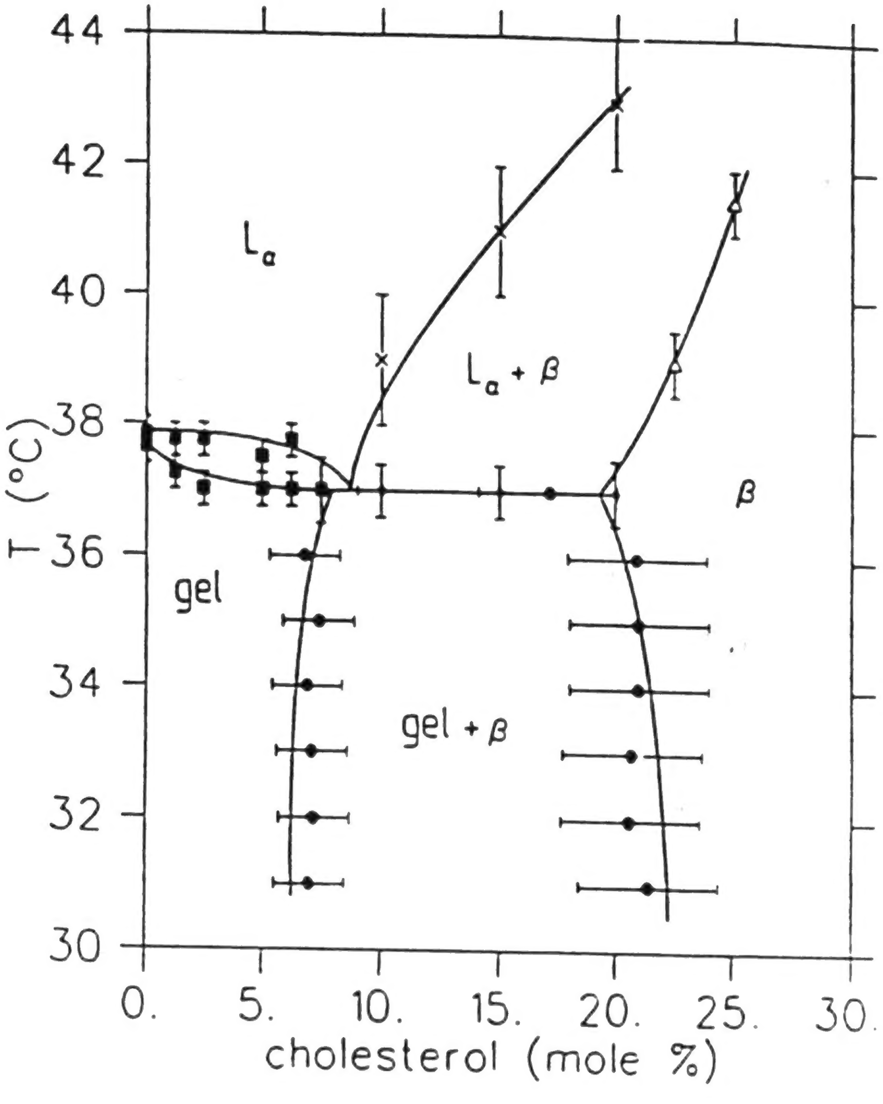

44
42
40
38
36
34
32
30
T (°C)
0.
5.
10.
15.
20.
25.
30.
cholesterol (mole %)
L_α
$L_\alpha + \beta$
β
gel
gel + β

Figure III-2. Raw representative DSC scans of annealed DPPC bilayers of various cholesterol molar concentrations. DPPC vesicles containing 1, 2, 3, 4, 6, 7, 8, 9, 11, 25, 30 and 50 mol% were also performed but are not illustrated here.

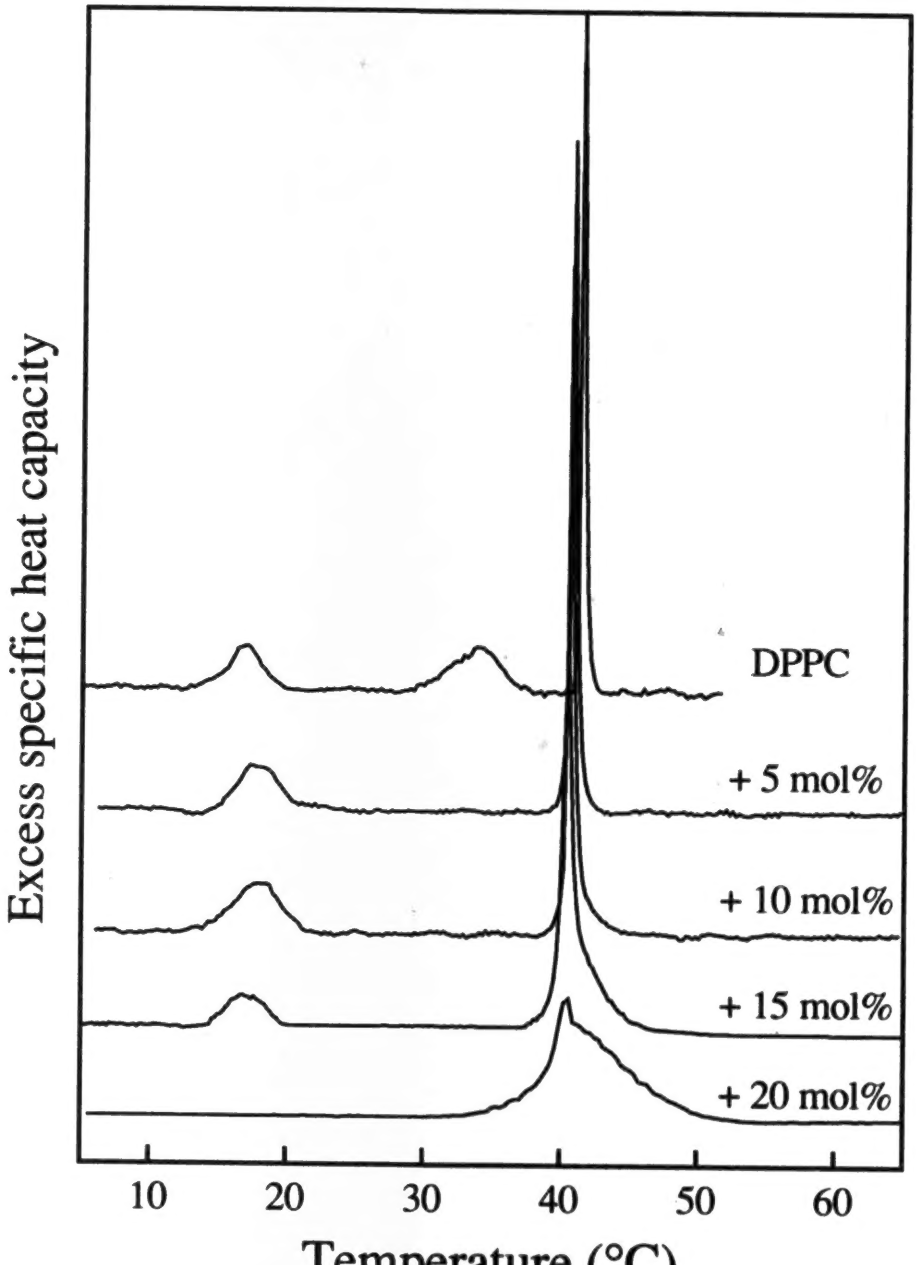

Excess specific heat capacity
DPPC
+ 5 mol%
+ 10 mol%
+ 15 mol%
+ 20 mol%
10
20
30
40
50
60
Temperature (°C)

Figure III-3. Representative decomposed endotherms of the main phase transition of DPPC with various amount of cholesterol. The solid line represents the overall endotherm while the dotted lines represent the sharp and broad components which are discernible by their transition temperatures and cooperativity (see text for discussion). Decompositions for 1, 3, 5, 7, 10, 15 and 20 mol% cholesterol were performed but are not shown here.

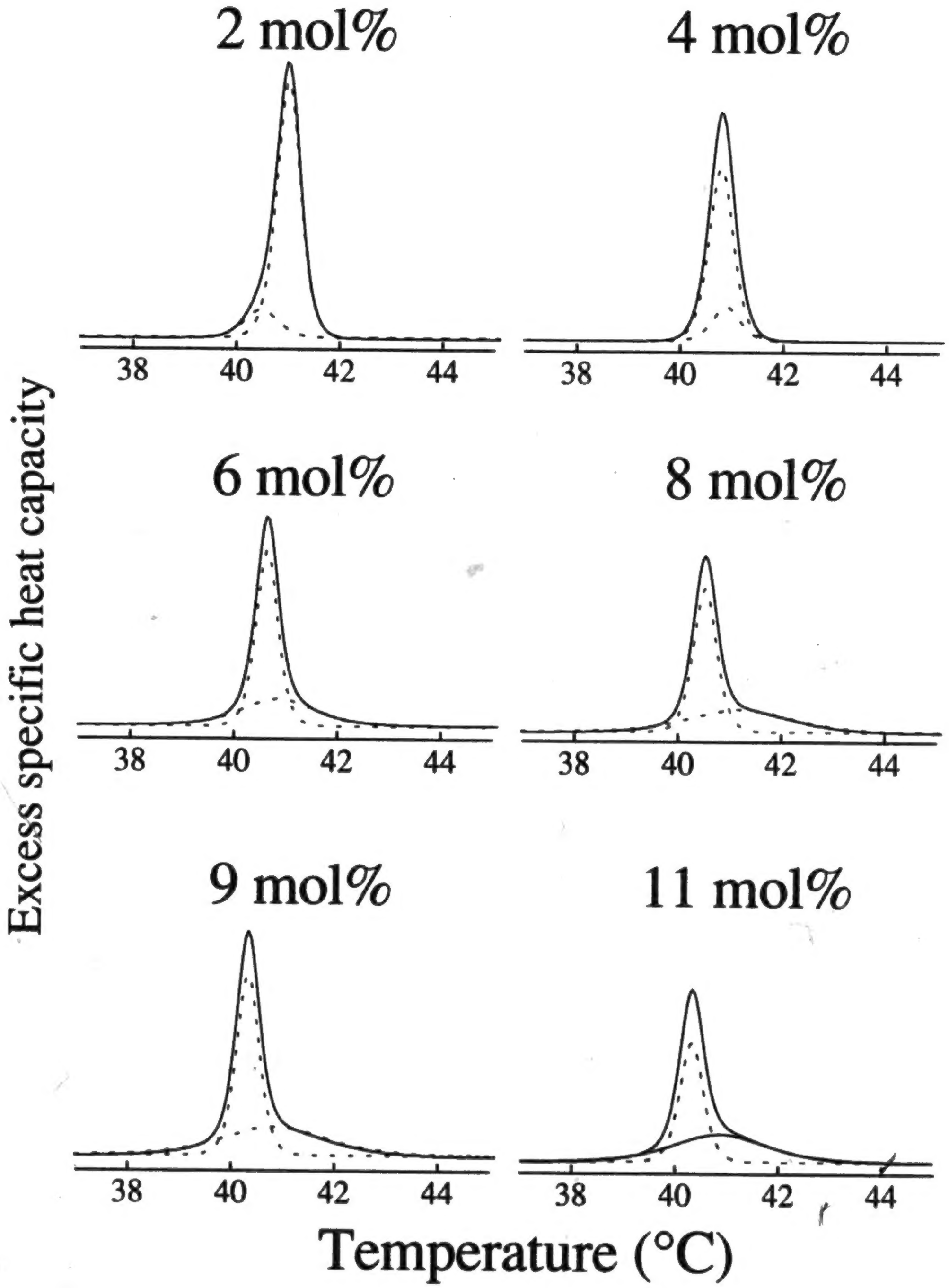

2 mol%
4 mol%
6 mol%
8 mol%
9 mol%
11 mol%
38
40
42
44
Excess specific heat capacity
Temperature (°C)

Figure III-4. (A); Effect of increasing amounts of cholesterol on the overall as well as broad and sharp component DPPC bilayer main transition chain-melting enthalpies. Legend for figure is as follows: overall (●); broad component (■); sharp component (Δ). (B) Plot of the DPPC/cholesterol bilayer sharp and broad melting component transition temperatures as a function of increasing cholesterol concentration up to 15 mol% cholesterol. Legend as in (A). (C) Effect of increasing levels of cholesterol on the DPPC bilayer main transition broad and sharp component $\Delta T_{1/2}$'s. Legend as in (A). Error falls within symbol size.

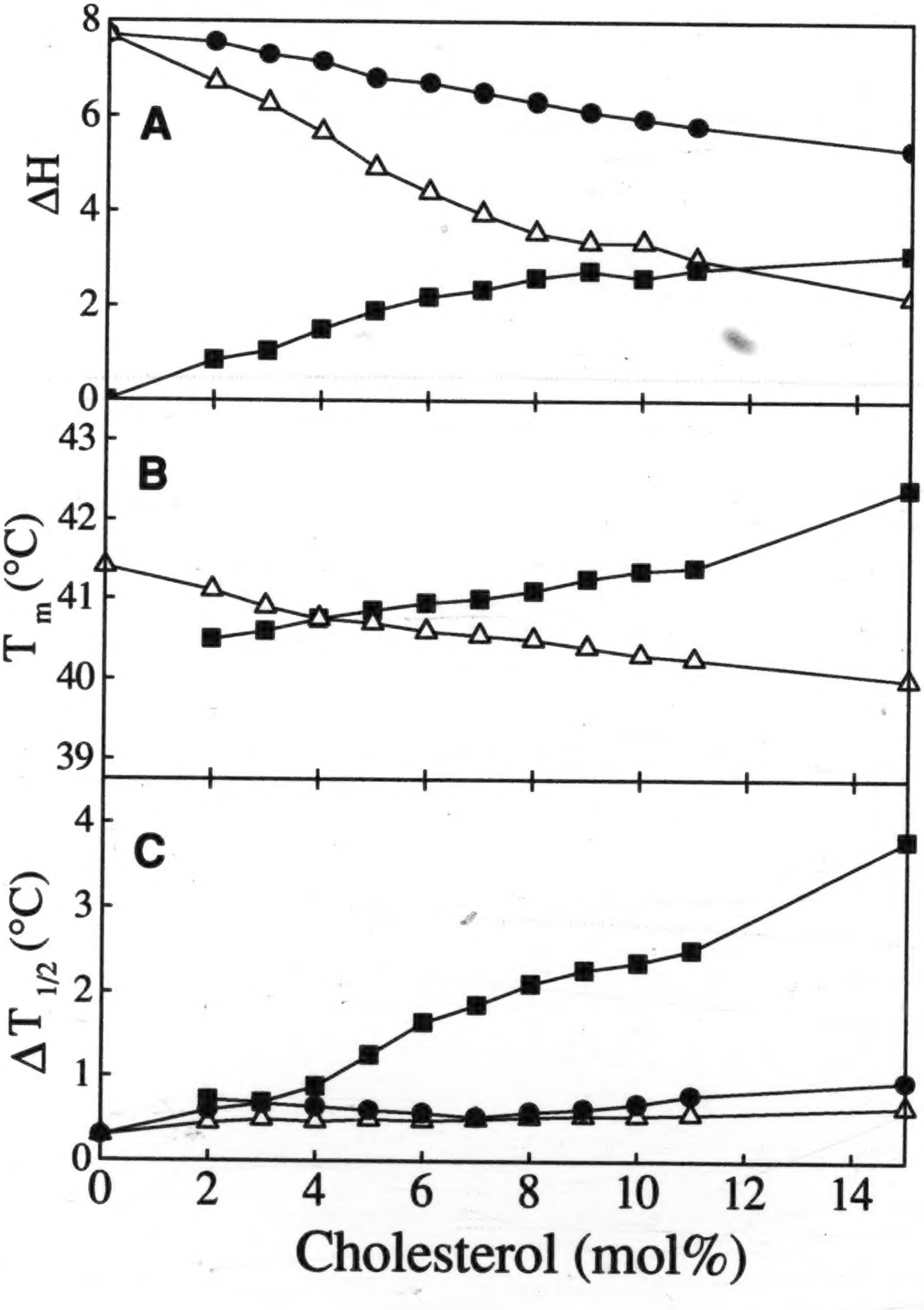

A
ΔH
0
2
4
6
8
B
T_m (°C)
39
40
41
42
43
C
$\Delta T_{1/2}$ (°C)
0
1
2
3
4
0
2
4
6
8
10
12
14
Cholesterol (mol%)

Figure III-5. Representative plots of the endotherm subtraction of pure DPPC from cholesterol-containing DPPC bilayers. The solid line represents the pure DPPC bilayer endotherm, the dotted line the cholesterol containing DPPC bilayer (top 2 mol%, bottom 5 mol%), and the difference between the curves is plotted by the dashed line at the bottom of each plot. DPPC/cholesterol samples performed but not shown are 3 and 4 mol% cholesterol.

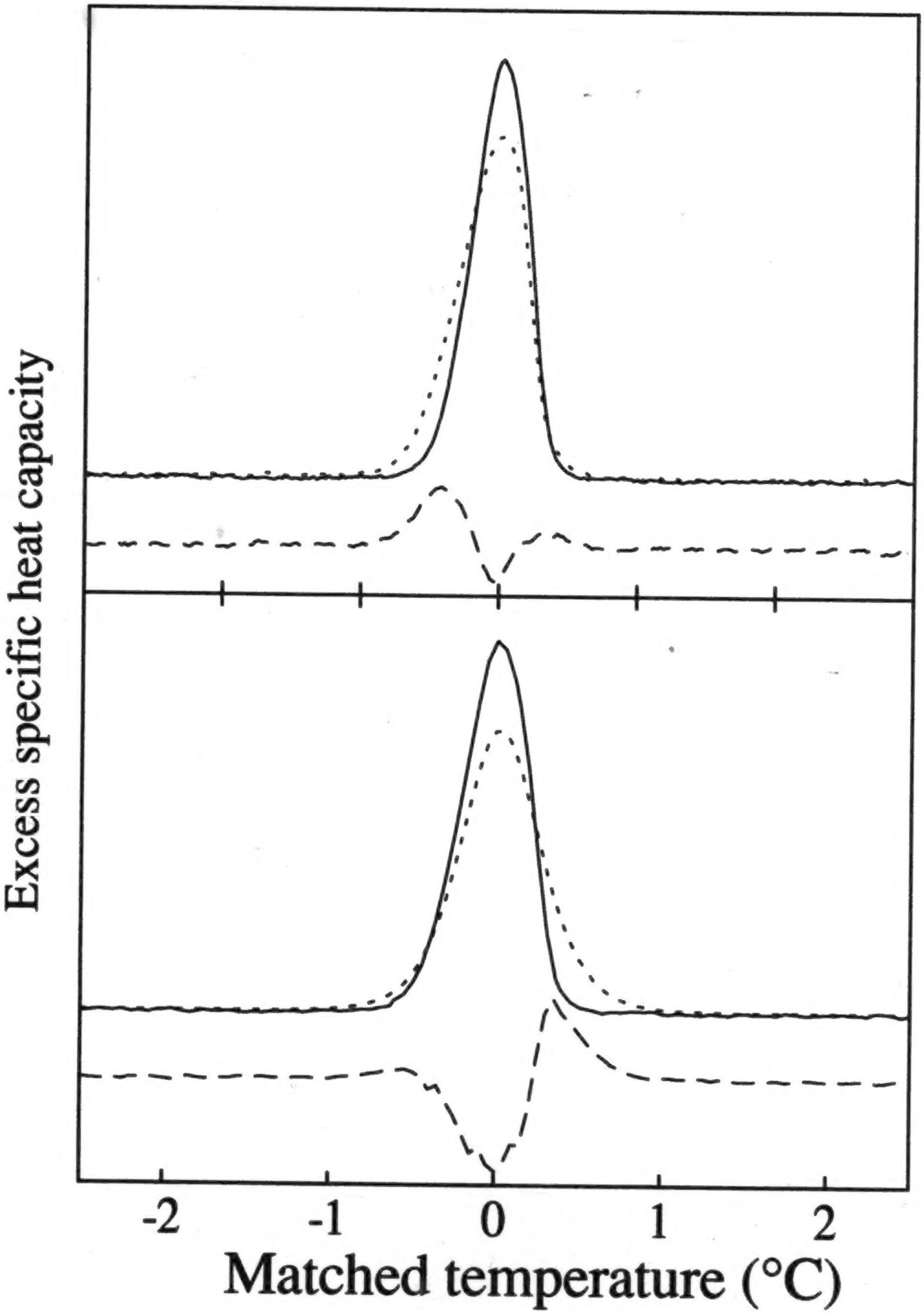

Excess specific heat capacity
-2
-1
0
1
2
Matched temperature (°C)

Figure III-6. Overall and decomposed plots of DMPC, DSPC and DAPC endotherms with various levels of cholesterol. The sharp and broad melting components are designated by the dotted lines, see text for a more detailed description. For cholesterol/DMPC mixtures the broadening due to cholesterol is such that the two scales are required to provide the resolution necessary to observe the sharp and broad components with increasing cholesterol. Cholesterol concentrations for each PC are as follows starting at the top: DMPC, 0, 2, 5, 10, 14 mol%; DSPC, 0, 2, 5, 10, 13 mol%; DAPC 0, 2, 5, 10, 13 mol%. With DMPC, between 14 and 17 mol% cholesterol, a third peak is evident which is not seen in any other PC. The origin of this peak is unclear.

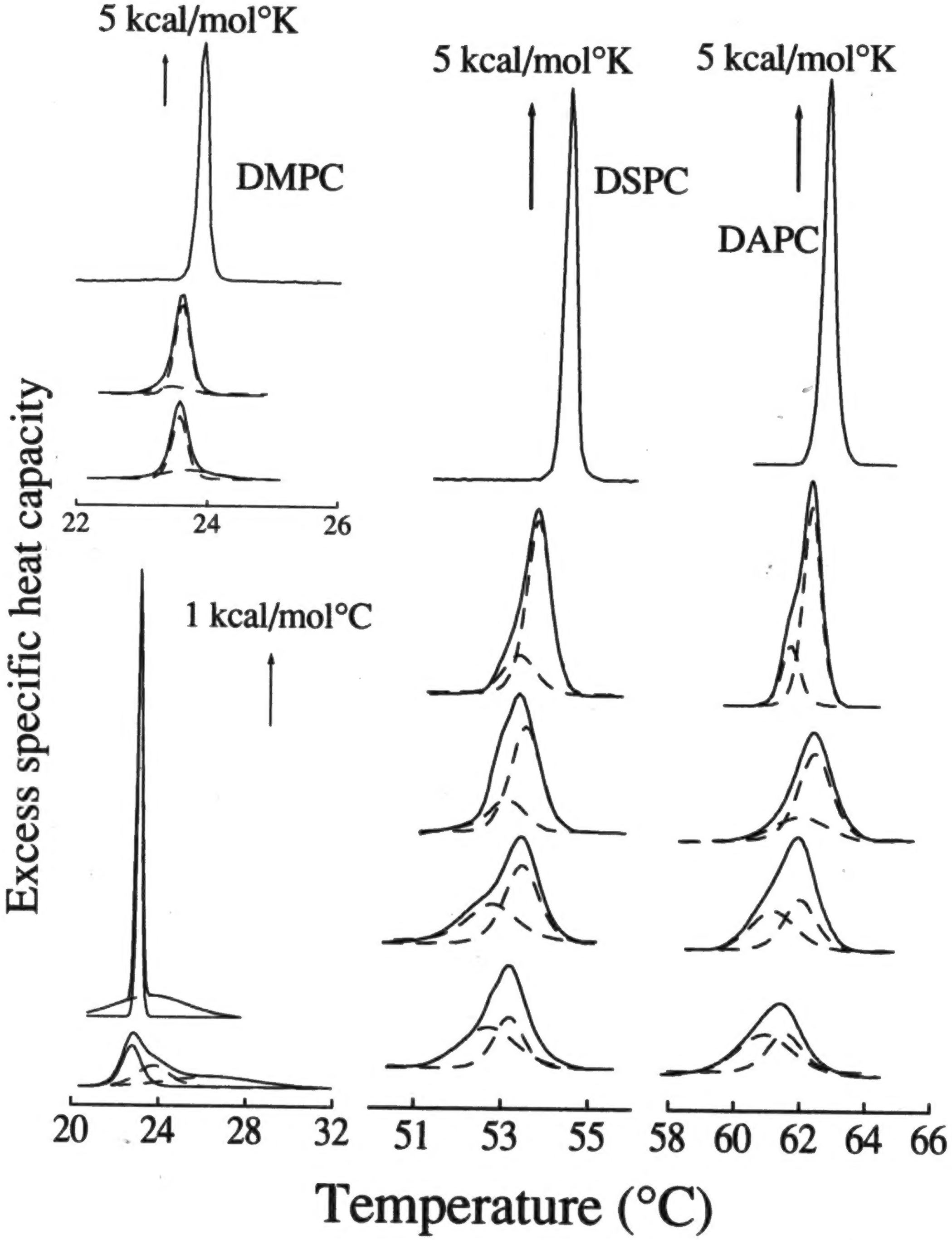

5 kcal/mol°K
DMPC
22
24
26
1 kcal/mol°C
20
24
28
32
5 kcal/mol°K
DSPC
51
53
55
5 kcal/mol°K
DAPC
58
60
62
64
66
Excess specific heat capacity
Temperature (°C)

Figure III-7. Overall $\Delta T_{1/2}$'s of DMPC, DPPC, DSPC and DAPC plotted as a function of cholesterol concentration. Legend for the figure is as follows: DMPC (■); DPPC (●); DSPC (◊); DAPC (∇).

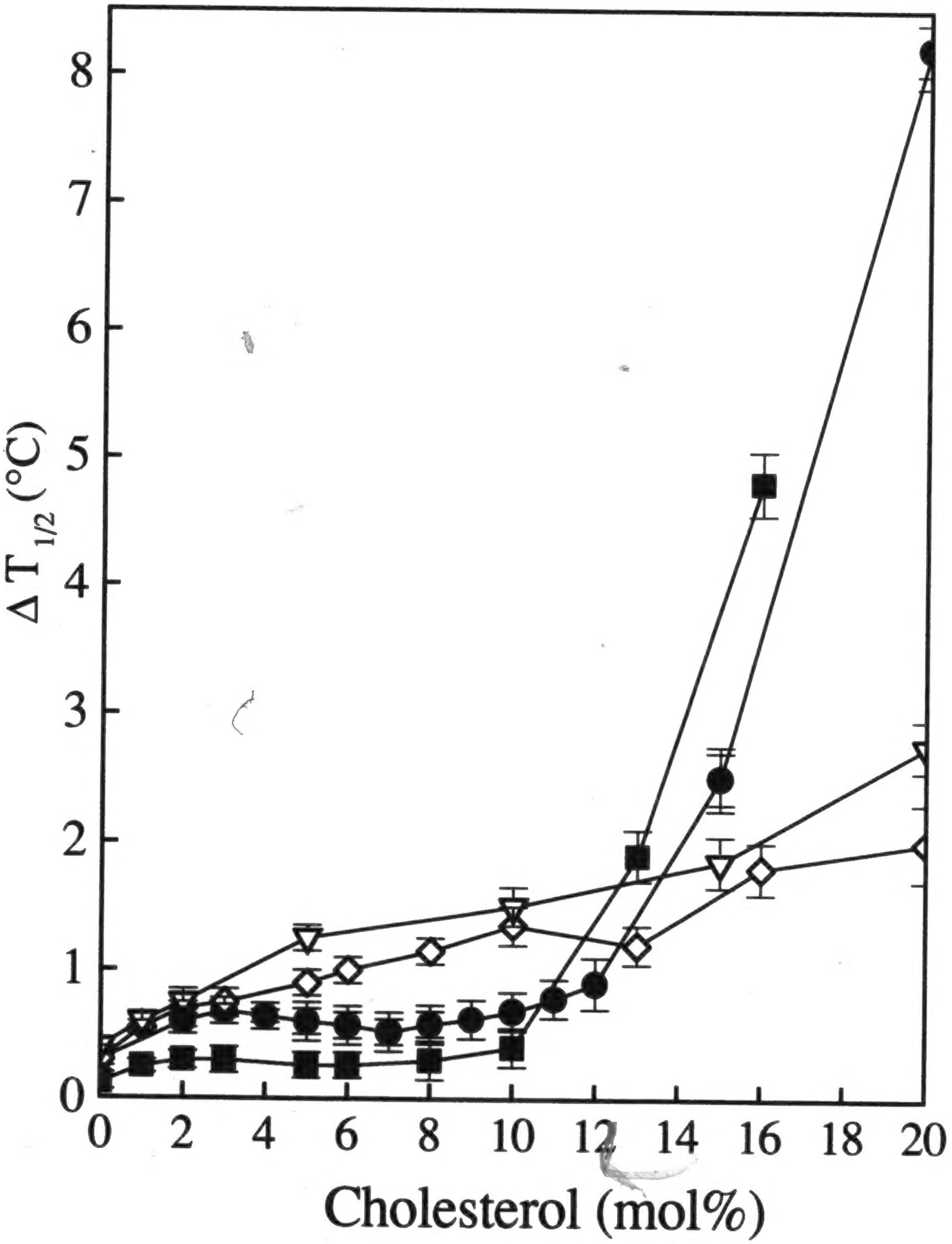
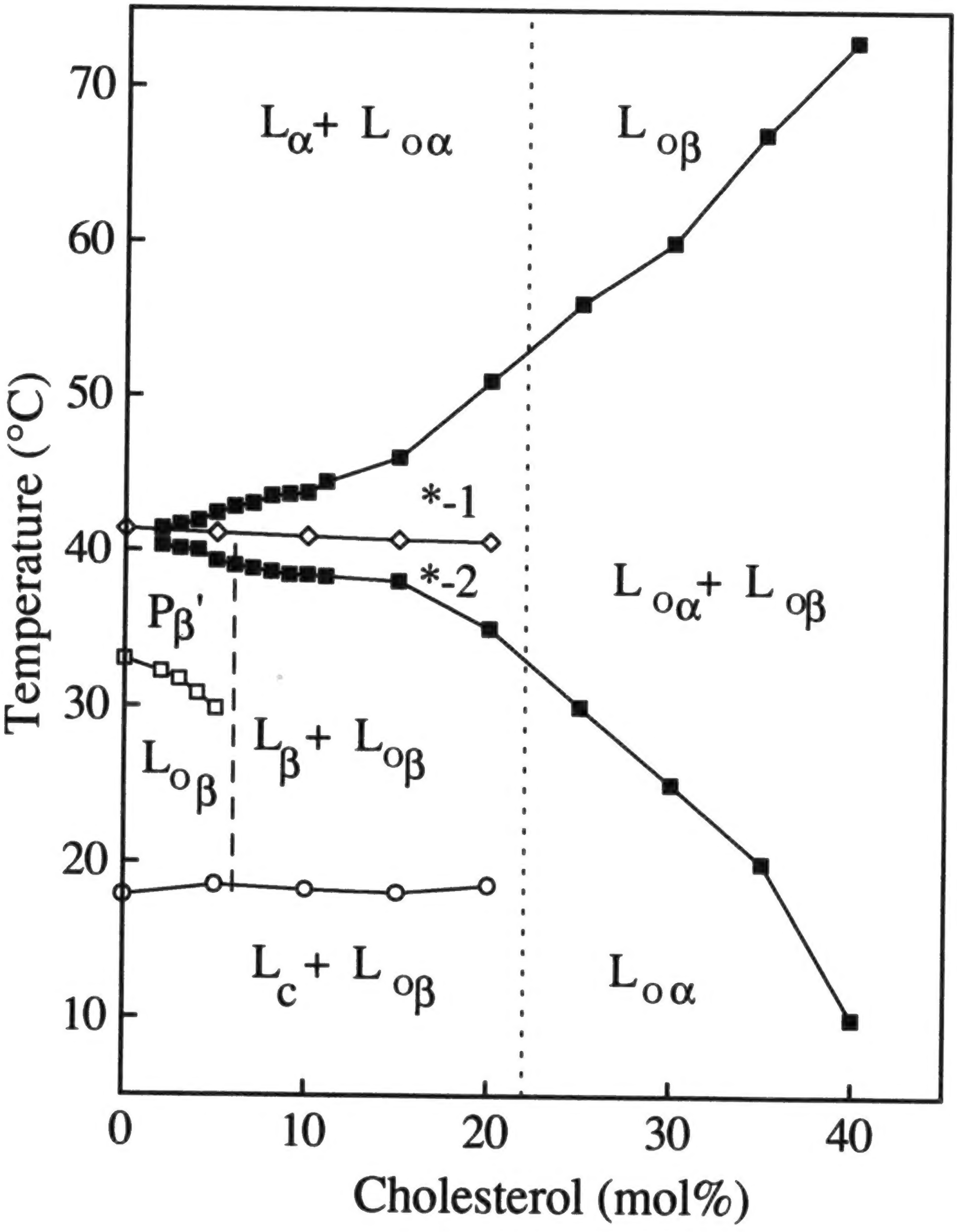

ΔT$_{1/2}$ (°C)
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
14
16
18
20
Cholesterol (mol%)

Figure III-8. Temperature/composition diagram for cholesterol/DPPC mixtures as a function of temperature and cholesterol concentration. A detailed description of the phase diagram is provided in the text. Briefly, the phase designations are as follows: L_c', the pure DPPC crystalline phase; L_β', the pure DPPC gel phase; $L_{o\beta}$, the cholesterol/DPPC liquid-ordered gel-like phase; $L_{o\alpha}$, the DPPC/cholesterol liquid-ordered liquid crystalline-like phase; P_β', the pure DPPC rippled phase; and L_α, the liquid-crystalline phase. The ′ designation indicates the lipid hydrocarbon chains in a particular phase are tilted to the bilayer normal. The transitions are marked as follows: the subtransition (○); pretransition (☐); pure lipid phase transition (◊); broad component phase transition boundaries (■). For $*^1$ and $*^2$ refer to text for a description of phase coexistence. If no error bars are shown, the degree of variability between runs is within the range of the symbol. Data for this phase diagram comes from this manuscript as well as (McMullen *et al.*, 1993).


Temperature (°C)
70
60
50
40
30
20
10
0
10
20
30
40
Cholesterol (mol%)
$L_\alpha + L_{o\alpha}$
$L_{o\beta}$
*-1
*-2
$L_{o\alpha} + L_{o\beta}$
P_β'
$L_{o\beta}$
$L_\beta + L_{o\beta}$
$L_c + L_{o\beta}$
$L_{o\alpha}$

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CHAPTER IV. COMPARATIVE DIFFERENTIAL SCANNING CALORIMETRIC AND FTIR AND ^{31}P-NMR SPECTROSCOPIC STUDIES OF THE EFFECTS OF CHOLESTEROL AND ANDROSTENOL ON THE THERMOTROPIC PHASE BEHAVIOR AND ORGANIZATION OF PHOSPHATIDYLCHOLINE BILAYERS[4]

INTRODUCTION

Cholesterol or a closely related sterol are major lipid components of the plasma membranes of most eukaryotic cells, and are also found in lower concentrations in many intracellular membranes (Nes and McKean, 1977). Cholesterol is also known to be an essential cellular component inasmuch as cholesterol synthesis or availability are required for the growth and viability of a variety of eukaryotic cells (Dahl and Dahl, 1988). Although cholesterol seems to have several different functions in eukaryotic cells, one of its primary roles is as a modulator of the physical properties of the plasma membrane phospholipid bilayer (Yeagle, 1988). Thus, a large number of studies of the effects of cholesterol incorporation on the properties of phospholipid monolayers and bilayers have been carried out utilizing a wide variety of physical techniques (see Demel and de Kruijff, 1976; Yeagle, 1985, 1988; Finean, 1990; Vist and Davis, 1990; McElhaney, 1992a). These studies have shown that cholesterol incorporation (i) broadens and eventually eliminates the cooperative gel to liquid-crystalline phase transition of phospholipid bilayers, (ii) decreases (increases) the area per molecule of liquid-crystalline (gel) state phospholipid monolayers, (iii) increases (decreases) the orientational order of the hydrocarbon chains of liquid-crystalline (gel) phospholipid bilayers; and (iv) decreases (increases) the passive permeability of phospholipid bilayers above (below) their gel to liquid-crystalline phase transition temperatures. At relatively high concentrations and higher temperatures, cholesterol appears to induce a "liquid-ordered" state in normally fluid phospholipid bilayers characterized by a mixture of gel and liquid-crystalline features. For example, in cholesterol-rich phospholipid bilayers the rates of phospholipid rotational (Sankaram and Thompson, 1990; Vist and Davis, 1990) and lateral (Alecio *et al.*, 1982; Kuo and Wade, 1979; Lindblom *et al.*, 1981; Rubenstein *et al.*, 1979) diffusion are comparable to those observed in the fluid phase of pure phospholipid bilayers, phospholipid hydrocarbon chain orientational order is intermediate between gel and liquid-crystalline of the pure lipid phases (Ipsen *et al.*, 1990; Vist and Davis, 1990), and the area compressibility modulus more closely resembles that of the gel phase of the pure phospholipid (Neeham *et al.*, 1988). The presence of cholesterol in biological membranes has also been shown to modulate a number of membrane functions, presumably via its effects on the properties of the phospholipid bilayer (Dahl and Dahl, 1988; Yeagle, 1988).

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[4] A version of this chapter has been published. McMullen, T.P.W., Lewis, R.N.A.H. and McElhaney, R.N. (1994) *Biophys. J.* 66: 741-752.

A number of researchers have investigated the effects of systematic variations in the structure and stereochemistry of the cholesterol molecule on the thermotropic phase behavior, organization and passive permeability of phospholipid bilayers (Demel and de Kruijff, 1976; Yeagle, 1985, 1988; McElhaney 1992a). In general, most structural and stereochemical alterations result in some loss of the ability of the cholesterol molecule to produce its characteristic effects on phospholipid bilayers. In general, sterols must possess an equatorially oriented C3-hydroxy group, a rigid planar fused ring system, and a flexible hydrocarbon side chain at C17 for maximum effect, while the degree of unsaturation of the ring system and the size of the alkyl side chain are of less importance. Interestingly, exactly the same structural features are required for exogenous sterols to support the maximum growth of sterol-auxotropic mycoplasma, yeast and mammalian cells (Dahl and Dahl, 1988; McElhaney, 1992b), confirming that one of the major roles of cholesterol in eukaryotic membranes is to regulate the physical properties of the lipid bilayer.

The cholesterol analogue androstenol (5-androsten-3-β-ol), also referred to as androsten, has proven to be very useful in studies of the effect of sterols on the physical properties of phospholipid monolayers and bilayers and on the growth of sterol auxotropic cells. Androstenol has exactly the same structure and stereochemistry as the parent cholesterol molecule except that it completely lacks the C17 alkyl side chain of the sterol nucleus. The incorporation of androstenol into phospholipid bilayers, however, seems to have little if any effect of the physical properties of model or biological membranes. Thus low-sensitivity DSC studies of androstenol-containing egg PC (Ladbrooke and Chapman, 1969) or SOPC (de Kruyff *et al.*, 1972) bilayers found only small effects of this sterol on the cooperativity and enthalpy of the gel to liquid-crystalline phase transition in comparison to cholesterol. Similarly, androstenol lacks the characteristic condensing effect exhibited by cholesterol in PC monolayers (Demel *et al.*, 1972a). Moreover, fluorescence polarization (Vincent and Galley, 1983) and FTIR (Senak *et al.*, 1992) spectroscopic studies of hydrocarbon chain order in DPPC bilayers found that androstenol was much less effective than cholesterol in reducing conformational disorder in the liquid-crystalline state. As well, androstenol, unlike cholesterol, is unable to significantly reduce the Rb^+, glycerol, or glucose permeability of egg PC bilayers (Demel *et al.*, 1972b) or the glycerol permeability of the human erythrocyte membrane (Bruckdorfer *et al.*, 1969). Finally, androstenol is unable to support the growth of a number of cholesterol-auxotropic mycoplasma, yeast and mammalian cells (Dahl and Dahl, 1988; McElhaney, 1992b). Thus the presence of an alkyl side chain at C17 seems to be a requirement for the cholesterol molecule to exert its characteristic effects in both model and biological membranes.

Singer and Finegold (1990a,b) recently carried out low-sensitivity DSC studies of the comparative effects of androstenol and cholesterol incorporation on the thermotropic phase behavior of a homologous series of PCs and PEs differing in the length of their hydrocarbon chains. Surprisingly, these workers report that the incorporation of increasing quantities of androstenol and cholesterol have virtually identical effects on the temperature, enthalpy and cooperativity of the chain-melting phase transitions of PCs and PEs, and that the sterol concentration required to completely abolish the gel to liquid-crystalline phase transition increases linearly with the length of the phospholipid hydrocarbon chains. However, these results are at odds with those of earlier calorimetric and other studies of the comparative effects of androstenol and cholesterol incorporation on phospholipid thermotropic phase behavior and organization in monolayer and bilayer model membranes. In addition, a marked linear dependence of sterol/phospholipid interaction stoichiometry on phospholipid hydrocarbon chain length cannot be explained by any of the current models of cholesterol/phospholipid packing. Finally, the use of a low-sensitivity DSC instrument with constant and relatively small phospholipid samples can result in failure to detect the less energetic and less cooperative phase transitions characteristic particularly of shorter chain phospholipids and high sterol concentrations, as we have demonstrated previously (McMullen *et al.*, 1993). For these reasons we have reinvestigated the comparative effects of androstenol and cholesterol incorporation on the thermotropic phase behavior of several members of a homologous series of linear saturated diacyl PCs utilizing high-sensitivity DSC and an experimental protocol insuring that broad, poorly energetic phase transitions can be accurately monitored. Indeed, using such an approach we have demonstrated that the effects of androstenol and cholesterol on PC thermotropic phase behavior are in some ways quite different. We have also utilized ^{31}P-NMR and FTIR spectroscopy to study the comparative effects of androstenol and cholesterol incorporation on the organization of the host PC bilayer in both the gel and liquid-crystalline states.

MATERIALS AND METHODS

The PCs used in these experiments were purchased from Avanti Polar Lipids and checked for purity by TLC using chloroform/methanol/ammonia (50:50:4, by vol.) as the developing solvent followed by spraying with sulfuric acid. Each PC gave a single spot on the developed TLC plates. The chain perdeuterated 14:0 PC (14:0 PC-d_{54}) was synthesized and purified using the procedures outlined in Lewis *et al.* (1985) except that perdeuterated myristic acid replaced myristic acid. The cholesterol was purchased from Fisher Chemicals and recrystallized twice from ethanol before use. The 5-androsten-3-β-ol was purchased from Steraloids. To ensure purity both sterol stock batches were checked by TLC using hexane/ether (7:3, by vol.) as the developing solvent. For each PC or sterol a known amount was then dissolved in chloroform to make stock solutions from which the sterol/PC dispersions would be quantitatively mixed. To ensure that the stock solutions gave homologous sterol/PC mixtures, we evaluated the thermotropic behavior and quantitative proportions of sterol/PC mixtures prepared from stock solutions of chloroform, chloroform/methanol (2:1), chloroform/methanol (1:2), and methanol. We found that the overall thermotropic phase behavior, as well as the quantitative differences in the predicted amount of PC or sterol within the mixtures, did not vary significantly with the solvent system. PC/sterol mixtures were then dried under N_2 and evaporated to dryness under vacuum overnight.

For DSC experiments the dried PC/sterol mixtures were dispersed and suspended with deionized water, heated to approximately 10-20 °C above the phase transition of the mixture, and then vortexed to give a multilamellar suspension. The DSC thermograms for the PC/sterol suspensions were recorded with a Hart high-sensitivity differential scanning calorimeter. Initially the scan rates used were 5 °C/hr for PC/sterol mixtures with less than 10 mol% sterol, while for increased amounts of sterol the scan rate was progressively increased to 20 °C/hr. In addition, the amount of PC used for DSC was progressively increased from 0.5 mg for pure PC bilayers to 5 mg for PC samples containing 50 mol% sterol. The Hart calorimeter has been calibrated using solid standards from Hart Scientific as well as aqueous lipid samples synthetically prepared within this laboratory using methods previously shown to provide highly pure samples (Lewis *et al.*, 1985). Sample runs were repeated at least three times to ensure reproducibility. After the DSC runs the samples were quantitated and checked for degradation using TLC with the developing solvent chloroform/methanol/ammonia (50:50:4) and sulfuric acid spray. No degradation was observed. The analysis and the deconvolution of the DSC endotherms was done using Microcal's Origin and DA-2 software. Briefly, the procedure for DSC deconvolution is based on the assumption that the observed thermograms can be approximated as a linear combination of multiple, independent two-state transitions as shown in Estep *et al.* (1978) and Mabrey *et al.* (1978) for sterol/PC mixtures. The curve broadening is expressed in terms of the van't Hoff enthalpy, which is evaluated by the equation $\Delta H_{vH}=4RT_m^2(c_{max}/\Delta q)$, where c_{max} is the excess specific heat capacity and Δq is the area under the curve. Although these equations provide a convincing description of the overall endotherm curves and have become a standard part of sterol/PC bilayer melting component analysis, it is not the only possible interpretation of the melting behavior.

For FTIR analysis the dried PC and sterol/PC dispersions were suspended in D_2O and prepared as described above for the DSC experiments. For the experiments involving perdeuterated sterol/DMPC mixtures, the hydration step used Milli-Q deionized water. The samples were then placed between CaF_2 windows with a 25-um spacer and mounted in a cell holder attached to a computer-controlled circulating water bath. FTIR spectra were recorded with a Digilab FTS-40 Fourier transform infrared spectrometer. The data was processed using computer programs developed by the National Research Council of Canada.

Pure PC or sterol/PC mixtures for ^{31}P-NMR were prepared as outlined for the DSC experiments. Proton-decoupled ^{31}P-NMR spectra were acquired with a Varian Unity-300 spectrometer operating at 121.42 MHz for ^{31}P. The data acquisition and data processing protocols were the same as the single-pulse, direct-excitation techniques described by Lewis *et al.* (1988). After completion of the NMR experiments the samples were checked for degradation by both TLC and DSC. No degradation or alterations in sample thermotropic behavior were observed.

RESULTS

Calorimetric studies of the thermotropic phase behavior of pure phosphatidylcholines

In this study the interactions of androstenol and cholesterol with a shorter chain, intermediate chain, and longer chain PC (DMPC, DPPC, and DSPC, respectively) were studied. High-sensitivity DSC heating scans of aqueous dispersions of these pure PCs are shown in Figures IV-1, IV-2, and IV-3 respectively. In the absence of sterol, these PC bilayers exhibit two endotherms on heating, a lower temperature, lower enthalpy pretransition and a higher temperature, higher enthalpy main transition. (Because these PC dispersions were not annealed at low temperature prior to heating, the lamellar crystalline (L_c') phase does not form and no subtransition is observed.) The pretransition arises from the conversion of the lamellar gel (L_β') phase to the rippled gel (P_β') phase and the main or chain-melting transition from a conversion of the P_β' phase to the lamellar liquid-crystalline (L_α) phase. Very similar behavior is noted for DMPC, DPPC and DSPC except that the temperature interval between these two transitions decreases as the length of the PC hydrocarbon chains increases. For a thorough description of the thermotropic phase behavior of these and other members of the homologous series of linear saturated PCs, the reader is referred to Lewis *et al.* (1987) and to the references cited therein.

Effect of androstenol and cholesterol on the pretransition

We have shown previously that the incorporation of low concentrations of cholesterol into PC bilayers progressively decreases the pretransition temperature and enthalpy in an approximately linear manner without significantly altering the cooperativity and that the pretransition becomes undetectable at cholesterol concentrations above 5 mol%, regardless of the hydrocarbon chain length of the PC molecule (McMullen *et al.*, 1993). Essentially similar results were obtained with androstenol in the present study, indicating that the alkyl side chain at C17 is not important for this particular sterol effect. However, since the incorporation of small quantities of free fatty acids and lysophospholipids (McElhaney, 1992a), or even transmembrane hydrophobic peptides (Zhang *et al.*, 1992), also abolishes the pretransition of PCs, the significance of this result is unclear and will not be discussed further.

Effect of androstenol and cholesterol on the main transition

Representative high-sensitivity DSC heating scans for DMPC dispersions containing increasing quantities of androstenol or cholesterol are presented in Figure IV-1. In general, the incorporation of increasing quantities of androstenol and cholesterol have rather similar effects on the DMPC endotherms. In both cases sterol incorporation decreases the enthalpy (Figure IV-4) and cooperativity (Figure IV-5) of the main phase transition of DMPC to a comparable extent and abolishes cooperative chain-melting at concentrations of 50 mol%. As well, the asymmetric DSC endotherms observed at androstenol concentrations of 1 to 20 mol% can be well fit by deconvolution into a sharp component, representing the melting of pure DMPC domains, and a broad component, representing the melting of androstenol-containing DMPC domains, just as previously demonstrated for cholesterol/DMPC mixtures (see McMullen *et al.*, 1993 and references cited therein). These results indicate that the effective stoichiometry of cholesterol/phospholipid and androstenol/phospholipid interactions are similar in DMPC bilayers. The one major difference between the effect of androstenol and cholesterol on DMPC thermotropic phase behavior is that androstenol decreases the phase transition temperature of the broad component of the DSC endotherm whereas cholesterol increases the phase transition temperature of this component (see Figure IV-6).

Representative high-sensitivity DSC heating scans of DPPC and DSPC dispersions containing increasing quantities of androstenol or cholesterol are presented in Figures IV-2 and IV-3, respectively. In contrast to DMPC bilayers, androstenol and cholesterol have rather different effects on the thermotropic phase behavior of these longer chain PCs. Specifically, at all but the lowest concentrations tested, the presence of androstenol produces a second, low-temperature DSC endotherm which is not observed in the cholesterol-containing dispersions. This low temperature endotherm is also seen on cooling scans and does not change in temperature or enthalpy over repeated scans. Moreover, the effects of androstenol and cholesterol on the higher temperature endotherm, which arises from the chain-melting transition of these longer chain PCs, are also quite different. Whereas increasing quantities of cholesterol progressively increase (decrease) the phase transition temperature of the broad component of the DSC endotherm of DPPC (DSPC) bilayers only slightly, increasing quantities of androstenol decrease the phase transition temperature of this component markedly in both of these PC bilayers (see Figure IV-6). As well, androstenol is less effective in reducing the enthalpy and cooperativity of the broad component of DPPC and especially DSPC bilayers in comparison to cholesterol (see Figures IV-4 and IV-5). Thus a cooperative albeit broad chain-melting phase transition is still observed in DPPC and especially in DSPC bilayers containing 50 mol% androstenol, whereas the presence of a comparable amount of cholesterol completely abolishes this phase transition. These results indicate that the effective stoichiometry of cholesterol/phospholipid and androstenol/phospholipid interactions are not the same in DPPC and especially in DSPC bilayers.

The DSC scans presented in Figure IV-2 and IV-3 for DPPC and DSPC, respectively, illustrate qualitatively the overall effect of the incorporation of increasing quantities of androstenol on the development and behavior of the low temperature endotherm observed only in the intermediate and longer chain PC bilayers. However, the thermodynamic characteristics of this transition vary not only with the level of androstenol added but also with the chain length of the host PC bilayer. For example, the T_m of the lower temperature transition varies in a complex manner with increasing androstenol concentration in the intermediate and longer chain PCs studied (Figures IV-2 and IV-3). In addition, as shown in Table IV-1, at comparable androstenol concentrations the low temperature endotherm occurs at a higher temperature compared to the pure PC, and becomes both more energetic and more cooperative as PC hydrocarbon chain length increases.

FTIR studies of androstenol/PC and cholesterol/PC mixtures

In these studies FTIR spectroscopy was used to assess the comparative effects of androstenol and cholesterol incorporation on the organization of the host lipid bilayer as a function of temperature. Thus temperature-induced shifts in the frequency of the CH_2 symmetric and asymmetric stretching band near 2850 cm^{-1} and 2920 cm^{-1} were used to monitor changes in the conformational state of the PC hydrocarbon chains, while alterations in the frequency of the CH_2 scissoring band near 1468 cm^{-1} indicated changes in the solid-state PC hydrocarbon chain packing (Mendelsohn and Mantsch, 1986; Mantsch and McElhaney, 1991). Because there were no significant changes in the maximum absorption frequency or shape of the PC ester carbonyl stretching bands at 1735 cm^{-1} or the P-O stretching bands upon sterol incorporation (data not presented), we will not discuss these regions of the FTIR spectra further. However, the incorporation of androstenol and cholesterol does have profound and at least quantitatively different effects on the organization of the hydrocarbon chains of the host PC bilayers at both high and low temperatures, as elaborated below.

Illustrated in Figure IV-7 are the variations in the frequency of the CH_2 asymmetric stretching band of pure DMPC, as well as androstenol/DMPC and cholesterol/DMPC binary mixtures containing 25 mol% sterol, as a function of temperature. The DSC endotherms of each sample are included to facilitate a comparison of the calorimetric and FTIR results. The concentration of 25 mol% sterol was chosen because at this level of incorporation domains of pure DMPC are no longer present. Thus all of the PC hydrocarbon chains are presumably interacting with sterol molecules but sterol-sterol interactions are minimized. However, qualitatively similar results were obtained with androstenol/PC and cholesterol/PC mixtures containing higher levels of sterol or when the CH_2 symmetric stretching band frequency was used to monitor hydrocarbon chain conformation (data not presented).

In the case of the pure DMPC dispersion, a sharp increase in the CH_2 asymmetric stretching frequency of about 3 cm^{-1} is observed at the temperature of the main phase transition as detected by DSC. Increases of this order of magnitude invariably accompany the melting of the hydrocarbon chains at the gel to liquid/crystalline phase transition of phospholipid bilayers (Mendelsohn and Mantsch, 1986; Mantsch and McElhaney, 1991). The androstenol/DMPC and the cholesterol/DMPC mixtures exhibit similar changes in CH_2 asymmetric frequency but these both occur over a much broader range of temperatures than for the pure DMPC dispersion. However, the temperature range over which most of the frequency change occurs in both systems again coincides fairly well with the DSC endotherms. This indicates that the DSC endotherms arise from the cooperative melting of the DMPC hydrocarbon chains in all three systems.

Plots of the frequency of the CH_2 asymmetric stretching band as a function of temperature for pure DSPC, and for androstenol/DSPC and cholesterol/DSPC mixtures containing 35 mol% sterol, are presented in Figure IV-8. To facilitate comparison with the calorimetric data, the corresponding DSC endotherms for each of these three systems is also illustrated. For DSPC alone, the CH_2 asymmetric stretching band frequency exhibits a large increase (~3.5 cm^{-1}) over a narrow range of temperatures centered near 55 °C, which corresponds well to the midpoint temperature of the main phase transition detected by DSC. As mentioned above, frequency changes of this magnitude in the CH_2 symmetric and asymmetric absorption bands invariably accompany the gel to liquid-crystalline phase transitions of phospholipid bilayers. In the case of the cholesterol/DSPC mixture, the increase in the CH_2 asymmetric stretching frequency is of comparable magnitude but occurs over a much broader range of temperatures and is centered near 53

°C, again in reasonable agreement with the DSC determination. However, the behavior of the androstenol/DSPC system is more complex than that of the DSPC and cholesterol/DSPC systems. Although the magnitude of the shift in the frequency of the CH_2 asymmetric stretching band is comparable to the former two systems, this shift in frequency appears to take place in two stages upon heating. A relatively small increase (~0.6 cm^{-1}) in CH_2 asymmetric stretching band frequency occurs at temperatures near 30 °C followed by a larger increase (~2.5 cm^{-1}) in band frequency near 50 °C. Moreover, the range of temperature over which this two-component frequency shift is observed (~23 °C) is somewhat less than that observed for the cholesterol/DSPC system (~ 30-35 °C). Again, the FTIR results for the androstenol/DSPC mixture correspond well to the DSC endotherms. In particular, these FTIR results indicate that the sharp, low temperature DSC endothermic event is accompanied by the introduction of a small amount of conformational disorder into the DSPC hydrocarbon chains, but that the higher temperature endothermic event corresponds to the major chain-melting phase transition of the host DSPC bilayer.

The absolute frequencies of the CH_2 symmetric and asymmetric stretching bands of any particular hydrocarbon chain are related to the degree of conformational disorder of that chain, with the introduction of increasing numbers of gauche conformers resulting in a progressively higher band frequency (Mendelsohn and Mantsch, 1986; Mantsch and McElhaney, 1991). Thus, in principle one can determine the relative effects of the incorporation of androstenol and cholesterol on the conformational disorder of the fatty acyl hydrocarbon chains of a phospholipid bilayer, in both the gel and liquid-crystalline states, by monitoring the CH_2 stretching band frequencies of sterol-containing phospholipid bilayers relative to those of the pure phospholipid. However, the data presented in Figures IV-7 and IV-8 can not be used for this purpose since the stretching bands of the CH_2 groups of the sterol ring system of androstenol and cholesterol, and of the CH_2 groups of the cholesterol alkyl side chain, overlap with those of the CH_2 groups of the phospholipid hydrocarbon chains. In order to overcome this problem, we monitored the CD_2 asymmetric stretching frequency of fully chain perdeuterated DMPC alone, and in the presence of either 25 mol% nonperdeuterated androstenol or cholesterol, and the results are presented in Figure IV-9. Note that at temperatures above the upper boundary of the calorimetrically detected main phase transitions (~50 °C), the frequency of the CH_2 asymmetric stretching band decreases somewhat upon the addition of androstenol to the DMPC bilayers but decreases to a much greater extent upon the addition of comparable amounts of cholesterol. These results are compatible with those of previous fluorescence polarization (Vincent and Galley, 1983) and FTIR (Senak *et al.*, 1992) spectroscopic studies of hydrocarbon chain order in DPPC bilayers, which found that androstenol was much less effective than cholesterol in reducing hydrocarbon chain conformational disorder in the liquid-crystalline state. The incorporation of androstenol and to a greater extent cholesterol into gel-state DMPC bilayers appears to increase DMPC hydrocarbon chain conformational order at temperatures below the onset of the calorimetrically detected main phase transition temperature (<10 °C). These findings are surprising in that previous monolayer film and ^{2}H-NMR studies of DPPC bilayers indicate that cholesterol disorders the hydrocarbon chains in the gel state. One would predict that both sterols, especially androstenol, would disorder the hydrocarbon chains of gel state DMPC bilayers due to the mismatch in effective hydrophobic lengths of the longer all-*trans* phospholipid hydrocarbon chains and the shorter sterol molecules. At present we have no good explanations for these results. We note, however, that an earlier FTIR spectroscopic study analyzing the effect of cholesterol on the PC CH_2 stretching bands also reports that cholesterol appears to decrease hydrocarbon chain conformational disorder of gel-state DPPC bilayers (Senak *et al.*, 1992).

Analysis of the CH_2 bending absorption band of the PC hydrocarbon chains as a function of temperature for pure DSPC, and for cholesterol/DSPC and androstenol/DSPC mixtures containing 35 mol% sterol, also demonstrated differences with respect to sterol incorporation (data not presented). For DSPC alone, the CH_2 bending absorption band is split into two components at temperatures below the pretransition temperature due to the strong lateral interactions of the PC hydrocarbon chains in the L_{β}' phase (see Lewis and McElhaney, 1990). Above the pretransition temperature these two components collapse into a single band due to the weaker lateral hydrocarbon chain interactions characteristic of the P_{β}' and especially of the L_{α} phase. In the cholesterol/DSPC system, no band splitting is observed even at low temperatures, presumably due to a weakening of hydrophobic chain interactions due to the disordering effect of cholesterol on the gel phase PC bilayers. However, such band splitting is observed in the androstenol/DSPC system, but only at temperatures below the low temperature endotherm observed by calorimetry. Significantly, the presence of comparable levels of androstenol in DMPC dispersions does not produce splitting of the hydrocarbon chain CH_2 scissoring band, as expected from its disordering effect on the hydrophobic core of these bilayers (data not presented). Thus the presence of CH_2 bending band splitting at low temperatures in the androstenol/DSPC mixtures indicates that domains of pure DSPC L_{β}' phase are present and thus that androstenol is largely immiscible in DSPC bilayers at those temperatures. Similarly, the disappearance of this band splitting at temperatures above that of the low temperature DSC endotherm in the androstenol/DSPC system indicates that domains of pure DSPC L_{β}' phase have largely disappeared, implying that androstenol is at least partially dispersed in the host PC bilayer at these higher temperatures, even though the DSPC hydrocarbon chains remain largely in their extended (unmelted) state.

^{31}P-NMR studies of androstenol/PC and cholesterol/PC mixtures

We employed ^{31}P-NMR spectroscopy to monitor the effect of androstenol and cholesterol on the mobility of the PC polar headgroup at the surface of the lipid bilayer, as well as to confirm that the androstenol/PC binary mixtures remain lamellar over the range of sterol concentrations and temperatures examined (see Seelig, 1978; Campbell *et al.*, 1979). Illustrated in Figure IV-10 are ^{31}P-NMR spectra of DMPC alone, as well as androstenol/DMPC and cholesterol/DMPC mixtures at sterol concentrations of 25 mol%, at temperatures both above and below their respective phase transitions. At temperatures above the upper boundary of the DSC endotherm of the sterol/PC mixtures, the spectra of pure DMPC and the sterol/DMPC mixtures are virtually identical. Specifically, all three spectra exhibit the shape and basal linewidth (~ 55 ppm) characteristic of the fast, axially symmetric motion of the phosphate polar headgroup in a lamellar liquid-crystalline bilayer (Seelig, 1978; Campbell *et al.*, 1979). Thus, although cholesterol, and to a lesser extent androstenol, order the hydrocarbon chains of the liquid-crystalline DMPC bilayer thereby decreasing the area occupied by the PC molecules, the incorporation of these sterols nevertheless actually increases the available surface area per PC headgroup in the bilayer. The net result is that the mobility of the PC polar headgroup, which is already high, remains so upon the incorporation of cholesterol or androstenol. In contrast, at temperatures below the lower boundary of the DSC endotherm of the sterol/DMPC mixtures, both sterols decrease the basal linewidth of the pure DMPC from ~ 85 ppm to ~60 ppm, indicating that both cholesterol and androstenol increase the mobility of the polar headgroup of gel phase DMPC to comparable extents. This effect is presumably because of both the disordering effect of both sterols on the DMPC hydrocarbon chains at low temperatures observed by other techniques (see Introduction) and by the polar headgroup spacing effect just discussed. A key point here is that comparable amounts of cholesterol and androstenol are probably present in the DMPC bilayer at low temperatures in order to produce spectral linewidths and shapes of comparable magnitude in the ^{31}P-NMR spectra.

The ^{31}P-NMR spectra of pure DSPC, as well as cholesterol/DSPC and androstenol/DSPC mixtures containing about 35 mol% sterol, at three different temperatures are presented in Figure IV-11. The ^{31}P spectra shown were taken at (A) 5°C (below the anomalous transition of androstenol/DSPC), (B) 35 °C (between the anomalous and chain-melting transitions of androstenol/DSPC), and (C) 60 °C (above the DSPC pure and androstenol/DSPC chain-melting transitions) and 70 °C (above the cholesterol/DSPC chain-melting transition). At 5 °C, both the ^{31}P power spectra for androstenol/DSPC and cholesterol/DSPC display a downfield shoulder and upfield peak indicative of a lamellar phase, but the basal and peak linewidth shown for the androstenol/DSPC bilayer is significantly larger (~25 ppm) and more like a gel-state pure PC bilayer than that shown for the cholesterol/DSPC bilayer at the same temperature. At 35°C, both lamellar spectra display substantially decreased basal and peak line widths such that the spectra for cholesterol/DSPC and androstenol/DSPC are now virtually superimposable. At temperatures above the chain melting transitions of the androstenol/DSPC and cholesterol/DSPC mixtures, both bilayer dispersions exhibit only slight decreases in basal and peak linewidths, indicating that the chain melting of androstenol/DSPC or cholesterol/DSPC bilayers has not significantly altered the rotational freedom of the phosphate groups at the bilayer interface. Because androstenol has a comparable effect to cholesterol in increasing polar headgroup mobility in gel-phase DMPC bilayers, it would appear that in DSPC bilayers below the anomalous calorimetric phase transition relatively small amounts of this sterol are interacting with the DSPC molecules. However, at temperatures above the anomalous transition but below the chain-melting phase transition, considerably more androstenol interacts with the host DSPC bilayer. These results, and the FTIR spectroscopic results just discussed, suggest that the low temperature phase transition observed by DSC results from a cooperative mutual solubilization of separate domains of pure androstenol and pure DSPC.

DISCUSSION

We believe that many of the differences between the effects of cholesterol and androstenol on the thermotropic phase behavior of PCs of different hydrocarbon chain lengths can be explained, at least qualitatively, by reference to the degree of hydrophobic mismatch between the effective lengths of these sterol molecules and the host PC hydrocarbon chains measured in a direction perpendicular to the bilayer plane (see Mouritsen and Bloom, 1984 and references cited therein). At the gel/liquid-crystalline phase transition temperature, the hydrophobic thickness of PC bilayers decreases by approximately one-third due to the introductions of *gauche* conformers into the fully extended, all-*trans* hydrocarbon chains. Thus the presence of another type of amphiphilic molecule, which does not undergo major temperature-induced changes in the length of its hydrophobic segment, can differently affect the relative stability of the gel and liquid-crystalline phases according to whether its intrinsic hydrophobic length more closely resembles that of the solid or fluid PC bilayer. At least to a first approximation, the relative stability of the gel and liquid-crystalline phases, and thus the chain-melting phase transition temperature, should be least affected when the hydrophobic length of the amphiphile equals the mean hydrophobic thickness of the bilayer (i.e., has an intrinsic hydrophobic length midway between that of the gel and liquid-crystalline phases). Amphiphiles with greater hydrophobic lengths would tend to differentially stabilize the gel phase of the host PC bilayer, thus increasing the gel to liquid-crystalline phase transition temperature, and vice versa. This hydrophobic mismatch effect has now been demonstrated to operate in synthetic peptide-PC (Zhang *et al.*, 1992) and cholesterol-PC (McMullen *et al.*, 1993) binary systems, and in peptide-cholesterol-PC ternary systems (Nezil and Bloom, 1992).

We have presented evidence elsewhere that the effective hydrophobic length of the cholesterol molecule in PC bilayers is about 17.5 Å (McMullen et al., 1993), a value close to that of the mean hydrophobic thickness PC bilayer with hydrocarbon chain lengths of 17 carbons. Thus the increase in the transition temperature of the cholesterol/PC broad component of the DSC endotherms observed for DMPC and DPPC bilayers, and the decrease observed for DSPC bilayer, can be explained by the hydrophobic mismatch theory. Similarly, molecular modeling indicates that the length of the androstenol molecule, which lacks the cholesterol alkyl side chain, should be about 12.5 Å, 5 Å shorter than the cholesterol molecule. Since the hydrophobic length of the androstenol molecule is considerably shorter than the mean hydrophobic thickness of the hydrocarbon chains of all the PCs studied here, we predict and indeed observe that the incorporation of androstenol should decrease the transition temperature of the broad component of the DSC endotherm in all cases. In fact hydrophobic mismatch theory predicts that the best match of hydrophobic lengths should occur for androstenol in PC bilayers with hydrocarbon chains 12 carbons long (DLPC), and indeed the temperatures of the sharp and the broad components of the DSC endotherms are very similar in androstenol/DLPC mixtures (data not presented).

The considerable and increasing degree of hydrophobic mismatch between the androstenol molecule and the gel phases of PC's with hydrocarbon chains of 16 carbons or longer may also explain the gel-phase immiscibility evident in these binary mixtures by DSC and by FTIR and ^{31}P-NMR spectroscopy. It is certain that in these systems the hydrophobic thickness of each monolayer of the gel-state bilayer significantly and progressively exceeds the hydrophobic length of the androstenol molecule as PC chain length increases, even considering that this sterol molecule disorders, and thus shortens, the hydrocarbon chains of the PC molecules with which it is in contact. This increasing degree of hydrophobic mismatch and thus gel phase immiscibility is probably responsible for the low-temperature DSC endotherm, which becomes increasingly prominent as the amount of androstenol present in the phospholipid dispersion or as the length of the hydrocarbon chains of the host PC bilayer increase (see Figures IV-2 and IV-3). Although the FTIR results indicate that the process giving rise to this rather cooperative endotherm involves some disordering of the gel state hydrocarbon chains of the host PC bilayer, most of the hydrocarbon chain conformational disorder is introduced during the high temperature DSC endotherm which corresponds to the main phase transition (see Figure IV-8). Note that in the androstenol/DSPC mixture containing 35 mol% sterol, the proportion of the total conformational disorder introduced during the lower temperature DSC endotherm (see Figure IV-8) appears to be much less than the fractional enthalpy of this endotherm (see Table IV-1). We thus suggest that the chain disordering accompanying the apparent dissolution of androstenol into the gel phase DSPC bilayers accounts for only a portion of the change in excess specific heat observed, and that phospholipid-androstenol mixing itself accounts for the remainder.

Since the hydrophobic length of the DSPC hydrocarbon chains in the gel state (~22.4 Å, see Zhang *et al.*, 1992) greatly exceeds the hydrophobic length of the androstenol molecule (~12.5 Å), it is not surprising that the temperature-induced introduction of androstenol in the gel-state DSPC bilayer would create some disorder in the phospholipid hydrocarbon chains. Moreover, even in the liquid-crystalline state of the pure DPPC bilayer, the hydrophobic length of the melted hydrocarbon chain would still exceed that of the androstenol molecule by 0.5-1.0 Å, and this hydrophobic mismatch is probably exaggerated by the ordering effect of this sterol on the fluid DPPC bilayer observed by FTIR spectroscopy. We therefore suggest that this increasing degree of hydrophobic mismatch between the liquid-crystalline PC bilayer and the androstenol molecule is responsible as well for the fluid phase immiscibility observed with these longer chain PC bilayers, which is manifested calorimetrically as the increasing large residual enthalpy observed at a sterol level of 50 mol% and as a smaller decrease in transition cooperativity relative to cholesterol. Conversely, the fact that the mean hydrophobic length of the androstenol molecule is intermediate between the hydrophobic length of gel and liquid-crystalline DMPC monolayers probably explains the lack of evidence for gel or liquid-crystalline phase immiscibility in this or in shorter-chain PC bilayers. Previous work on synthetic hydrophobic transmembrane α-helical peptide/PC (Zhang et al., 1992) and on natural membrane protein/PC (Lewis and Engelman, 1983; Riegler and Mohwald, 1986) binary mixtures has shown that lipid/protein phase separation can occur in very long chain PCs host bilayers.

It is clear from this and other recent studies that the interaction of sterols with phospholipid bilayers is a complex process which can be influenced by temperature, by sterol concentration, and by the structure of the phospholipids and sterols themselves. Clearly, additional studies, utilizing modern structural, thermodynamic, and spectroscopic techniques, and sterols and phospholipid molecules whose structures are systematically varied, will be required to fully understand these interactions. Such studies are currently underway in this laboratory.

Figure IV-1. Representative DSC scans of unannealed DMPC bilayers with various concentrations of cholesterol or androstenol. Cholesterol/DMPC thermograms are represented by the dotted lines while the androstenol/DMPC traces are represented by the solid lines just below. The sterol concentrations represented by the DSC traces are shown in the figure. The highest androstenol concentration used was 45 mol% while for cholesterol the highest concentration examined was 50 mol%. DSC scans containing 1, 2, 15, 25, 30 and 40 mol% cholesterol and 15, 25, and 30 mol% androstenol were also performed but not shown. Endotherms are not corrected for scan rate or mass of sample. (Cholesterol/DMPC traces obtained from McMullen *et al.*, 1993.)

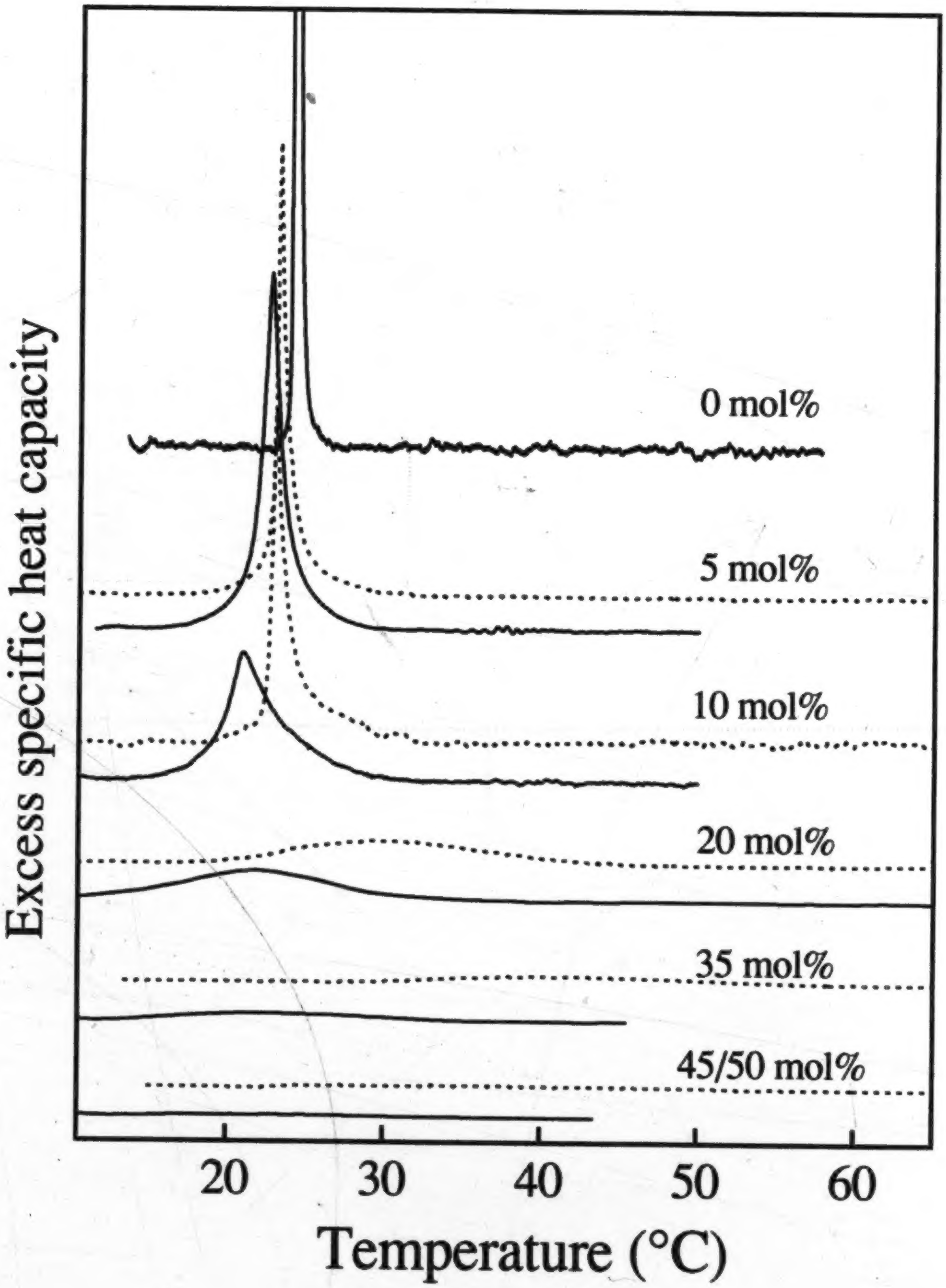
Excess specific heat capacity
0 mol%
5 mol%
10 mol%
20 mol%
35 mol%
45/50 mol%
20
30
40
50
60
Temperature (°C)

Figure IV-2. Representative DSC scans of unannealed DPPC bilayers with various concentrations of cholesterol or androstenol. Cholesterol/DPPC thermograms are represented by the dotted lines while the androstenol/DPPC mixtures represented by the solid lines just below. The sterol concentrations are shown in the figure for each set of DSC traces. The highest androstenol concentration used was 45 mol% while for cholesterol the highest concentration examined was 50 mol%. DSC scans performed but not shown are; 1, 2, 3, 4, 6, 15, 25, 30 and 40 mol% for cholesterol/DPPC, and 15, 25, and 30 mol% for androstenol/DPPC mixtures. Endotherms are not corrected for scan rate or mass of sample. (Cholesterol/DPPC traces obtained from McMullen *et al.*, 1993.)

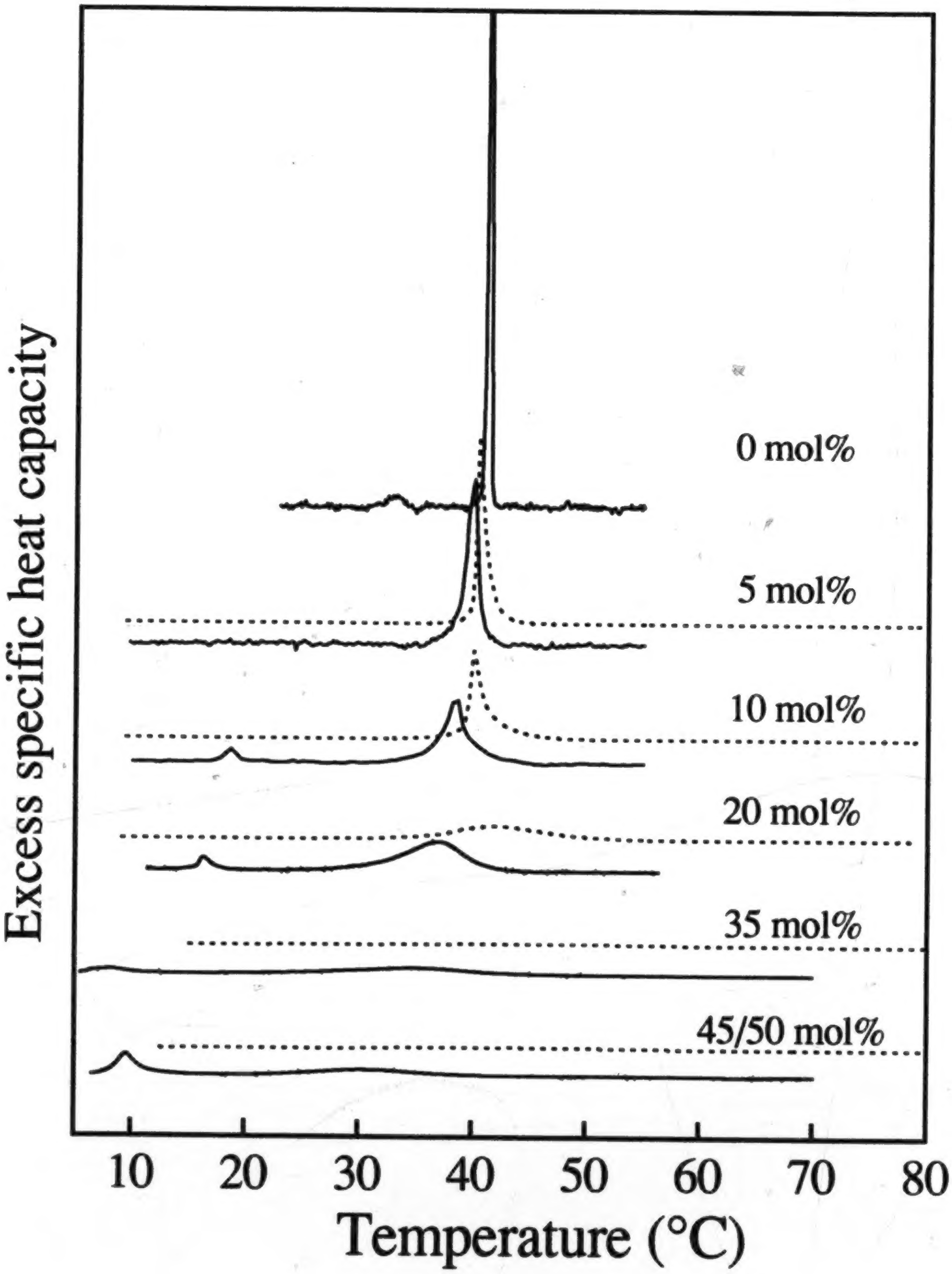
0 mol%
5 mol%
10 mol%
20 mol%
35 mol%
45/50 mol%
Excess specific heat capacity
10
20
30
40
50
60
70
80
Temperature (°C)

Figure IV-3. Representative DSC scans of unannealed DSPC bilayers with various concentrations of cholesterol or androstenol. Cholesterol/DSPC thermograms are represented by the dotted lines while the androstenol/DSPC mixtures represented by the solid lines just below. The sterol concentrations are shown in the figure for each set of DSC traces. The highest androstenol concentration used was 45 mol% while for cholesterol the highest concentration examined was 50 mol%. DSC scans performed but not shown are; 1,2,15,25,30 and 40 mol% for cholesterol/DSPC, and 15,25, and 30 mol% for androstenol/DSPC mixtures. Endotherms are not corrected for scan rate or mass of sample.(Comparative cholesterol/DSPC data obtained from McMullen *et al.*, 1993.)

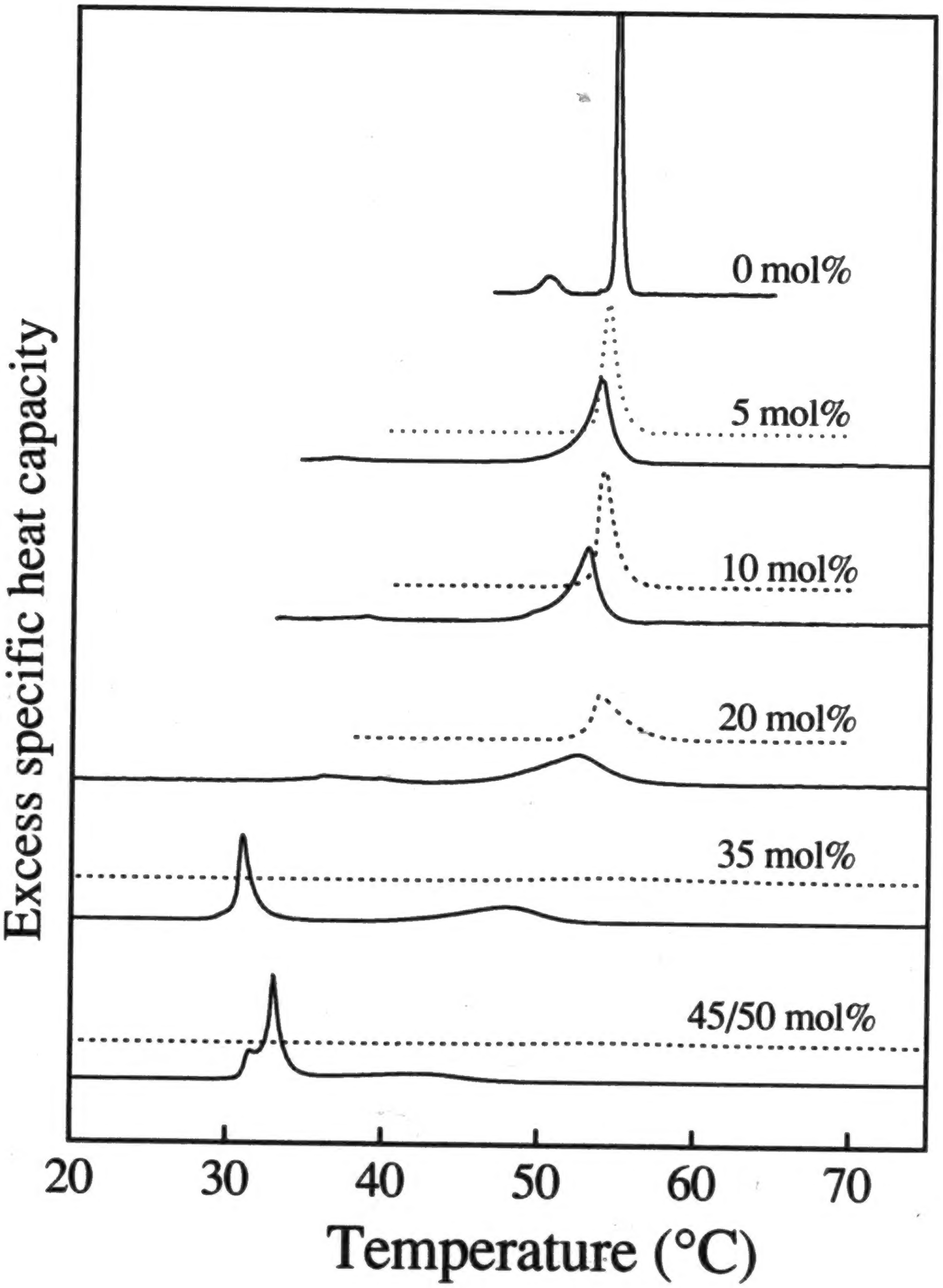
0 mol%
5 mol%
10 mol%
20 mol%
35 mol%
45/50 mol%
Excess specific heat capacity
20
30
40
50
60
70
Temperature (°C)

Figure IV-4. A comparison of the DMPC, DPPC and DSPC main transition enthalpies as a function of increasing sterol concentration. The legend for the figure is as follows: androstenol/DMPC overall (◆), androstenol/DMPC sharp-melting component (■), androstenol/DMPC broad-melting component (Δ), cholesterol/DMPC overall (◊), cholesterol/DMPC sharp-melting component (❑), cholesterol/DMPC broad-melting component, (❍), androstenol/DPPC overall (▼), cholesterol/DPPC overall (∇), androstenol/DSPC overall (▲), and cholesterol/DSPC overall (❍). (Cholesterol/CnPC data obtained from McMullen *et al.*, 1993.]

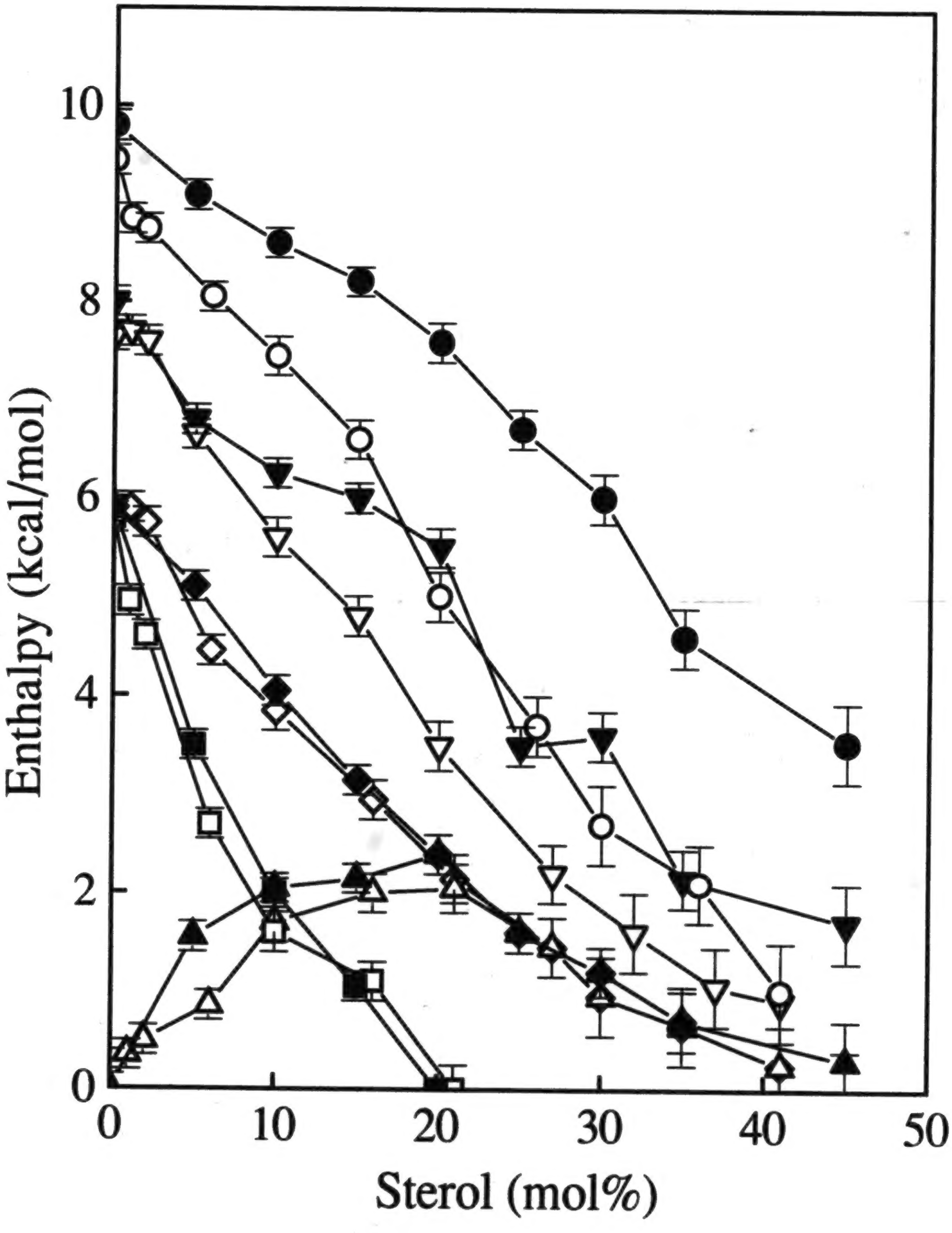
Enthalpy (kcal/mol)
Sterol (mol%)
0
2
4
6
8
10
0
10
20
30
40
50

Figure IV-5. Dependence of the DMPC, DPPC and DSPC broad component transition $\Delta T_{1/2}$'s on increasing sterol concentration. Legend for the figure is as follows: androstenol/DMPC (◆), cholesterol/DMPC (◊), androstenol/DPPC (▼), cholesterol/DPPC (▽), androstenol/DSPC (●), and cholesterol/DSPC (○). (Data for cholesterol/CnPC mixtures from McMullen *et al.*, 1993.)

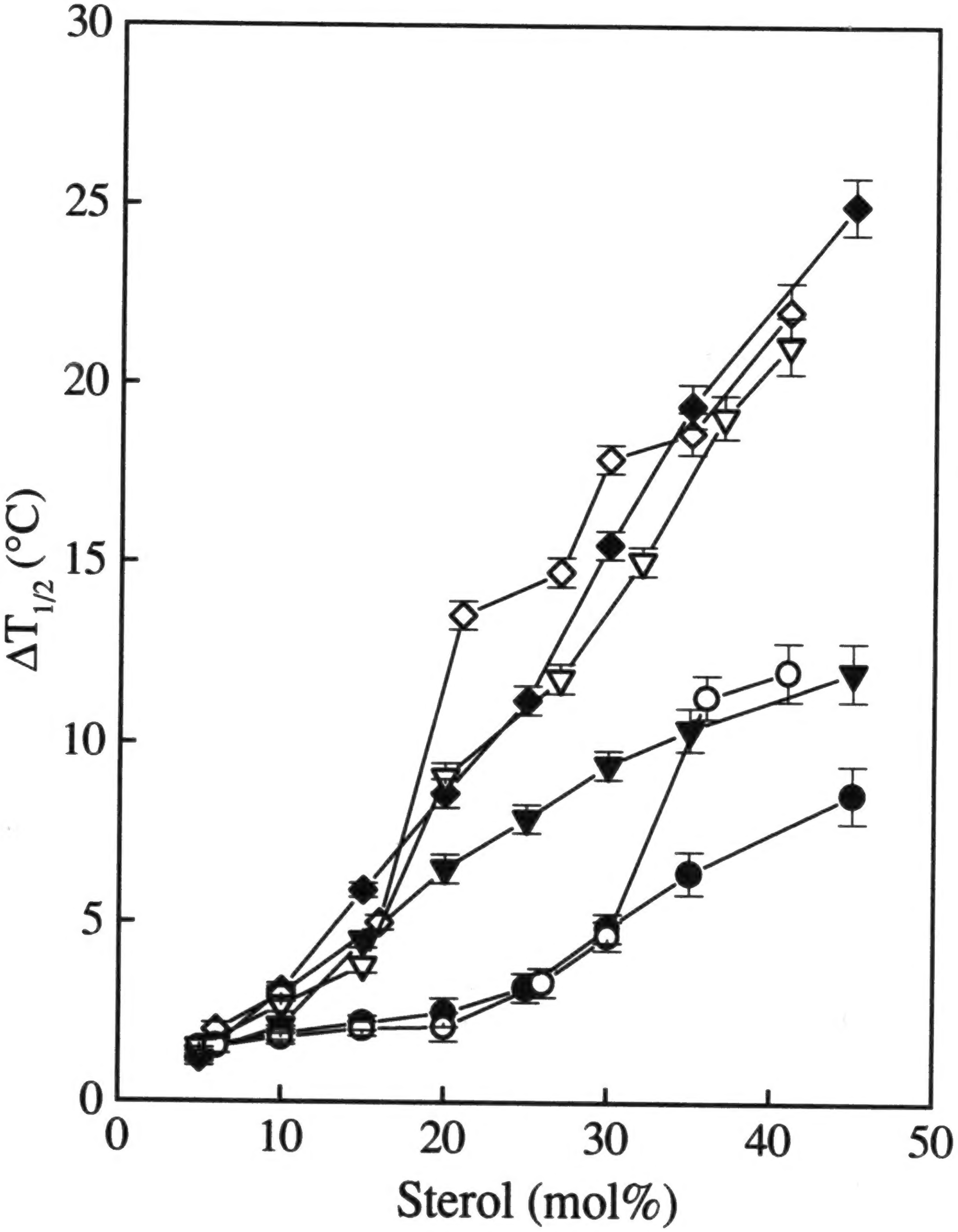
ΔT_{1/2} (°C)
0
5
10
15
20
25
30
Sterol (mol%)
0
10
20
30
40
50

Figure IV-6. Plot of the broad component transition temperature shift as a function of increasing sterol concentration for short, intermediate and long chain PCs. Legend for PC's in figure: androstenol/DMPC (◆), cholesterol/DMPC (◊), androstenol/DPPC (▼), cholesterol/DPPC (∇), androstenol/DSPC (●), cholesterol/DSPC (○). Temperatures are corrected for scan rate. (Comparative cholesterol/CnPC data obtained from McMullen *et al.*, 1993.)

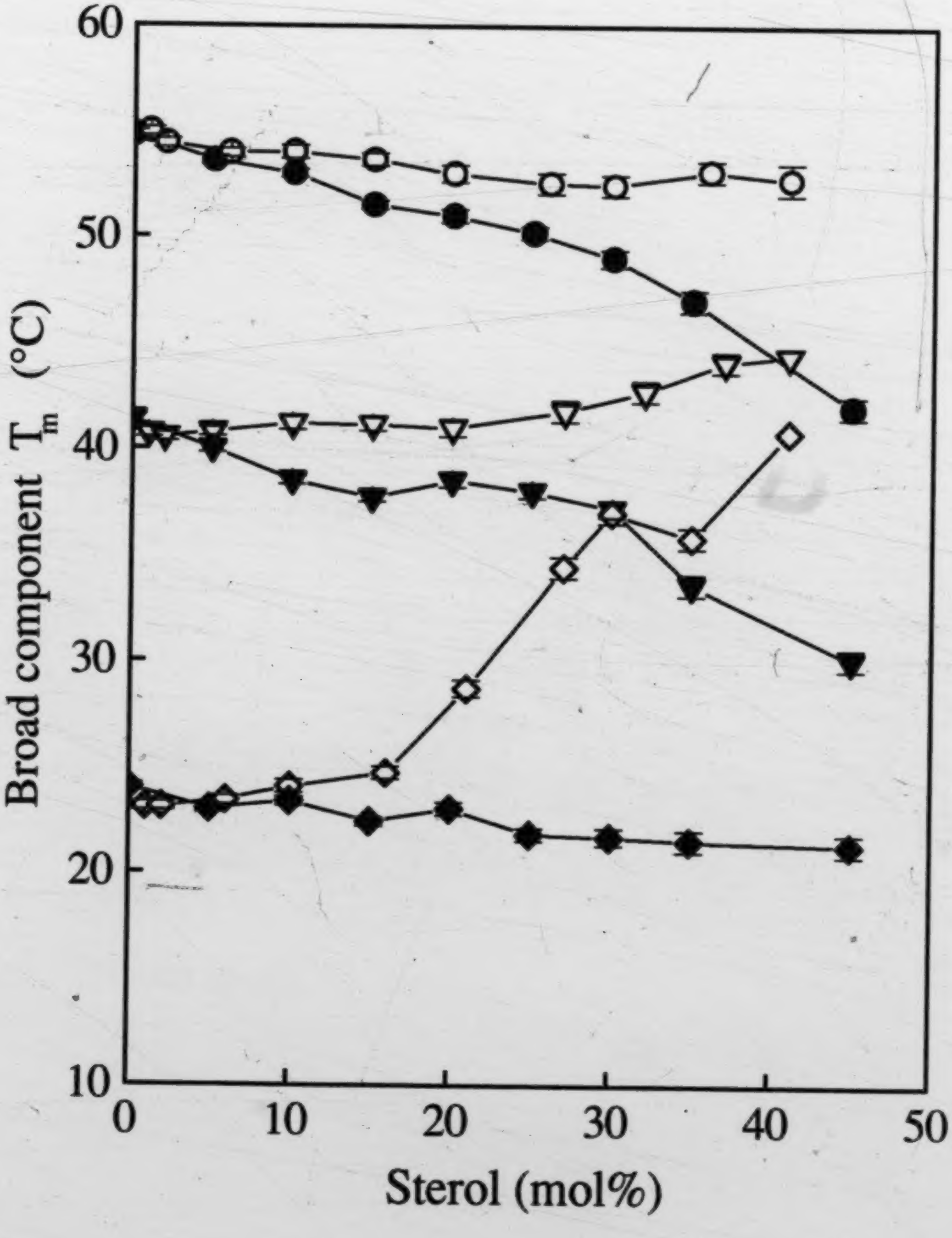
60
50
40
30
20
10
0
10
20
30
40
50
Broad component T_m (°C)
Sterol (mol%)

Figure IV-7. A comparison of FTIR and DSC data for: **A**, pure DMPC; **B**, androstenol/DMPC (25 mol%); and C, cholesterol/DMPC (25 mol%) bilayers. Along the top are the plots of the CH_2 asymmetric stretching absorption maxima as a function of temperature. The corresponding DSC traces are shown for comparative purposes directly below. The CH_2 symmetric stretching spectra absorption maxima are not shown but gave qualitatively very similar results. The endotherms are corrected for scan rate but are not corrected for sample mass.

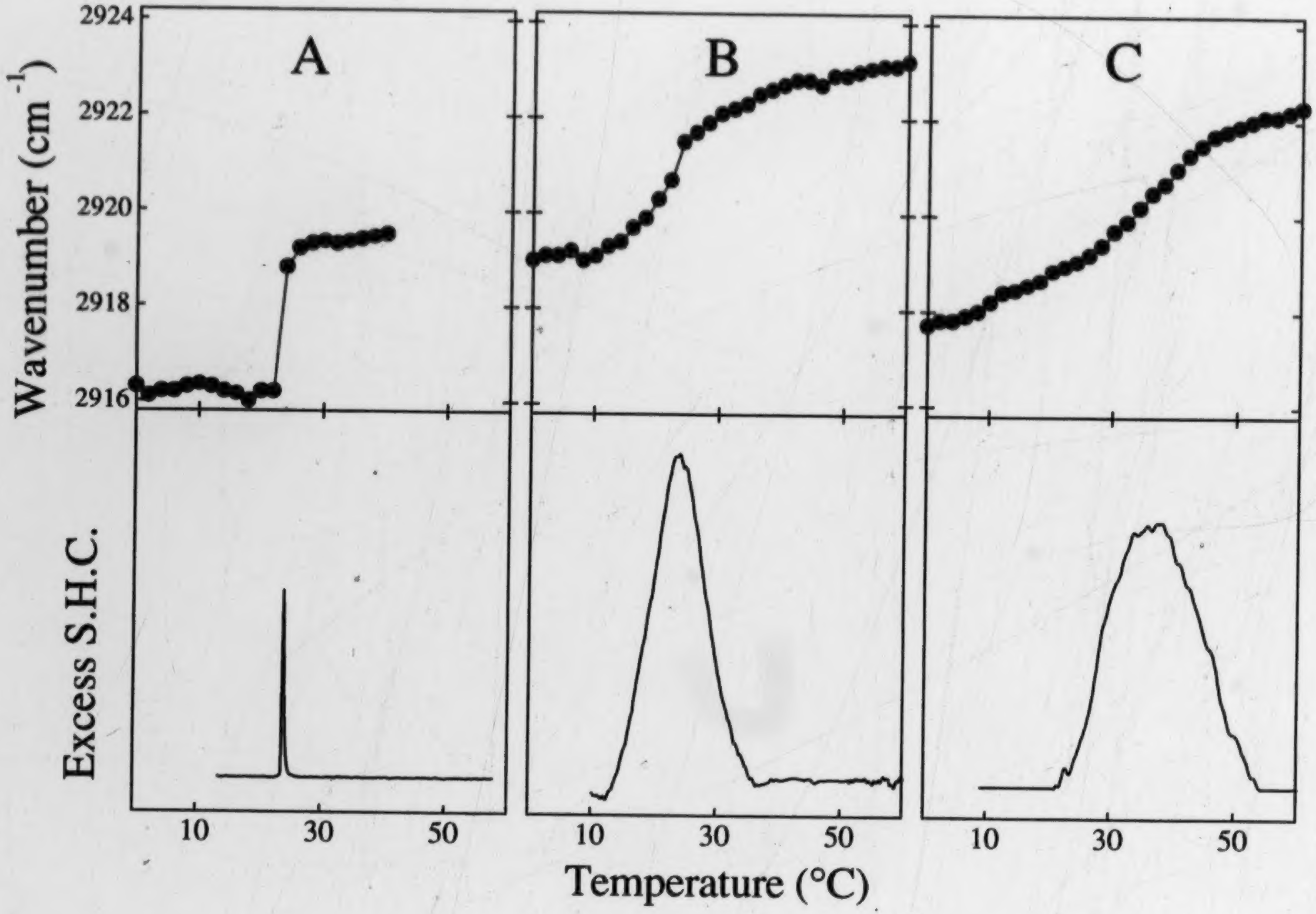
A
B
C
Wavenumber (cm $^{-1}$)
2924
2922
2920
2918
2916
Excess S.H.C.
10
30
50
Temperature (°C)

Figure IV-8. A comparison of FTIR and DSC data for: **A**, pure DSPC; **B**, androstenol/DSPC (35 mol%); and **C**, cholesterol/DSPC (35 mol%) bilayers. Along the top are plots of the CH_2 asymmetric stretching absorption maxima as a function of temperature. The corresponding DSC traces are shown for comparative purposes directly below. The CH_2 symmetric stretching spectra absorption maxima are not shown but gave qualitatively very similar results. The endotherms are corrected for scan rate but are not corrected for sample mass.

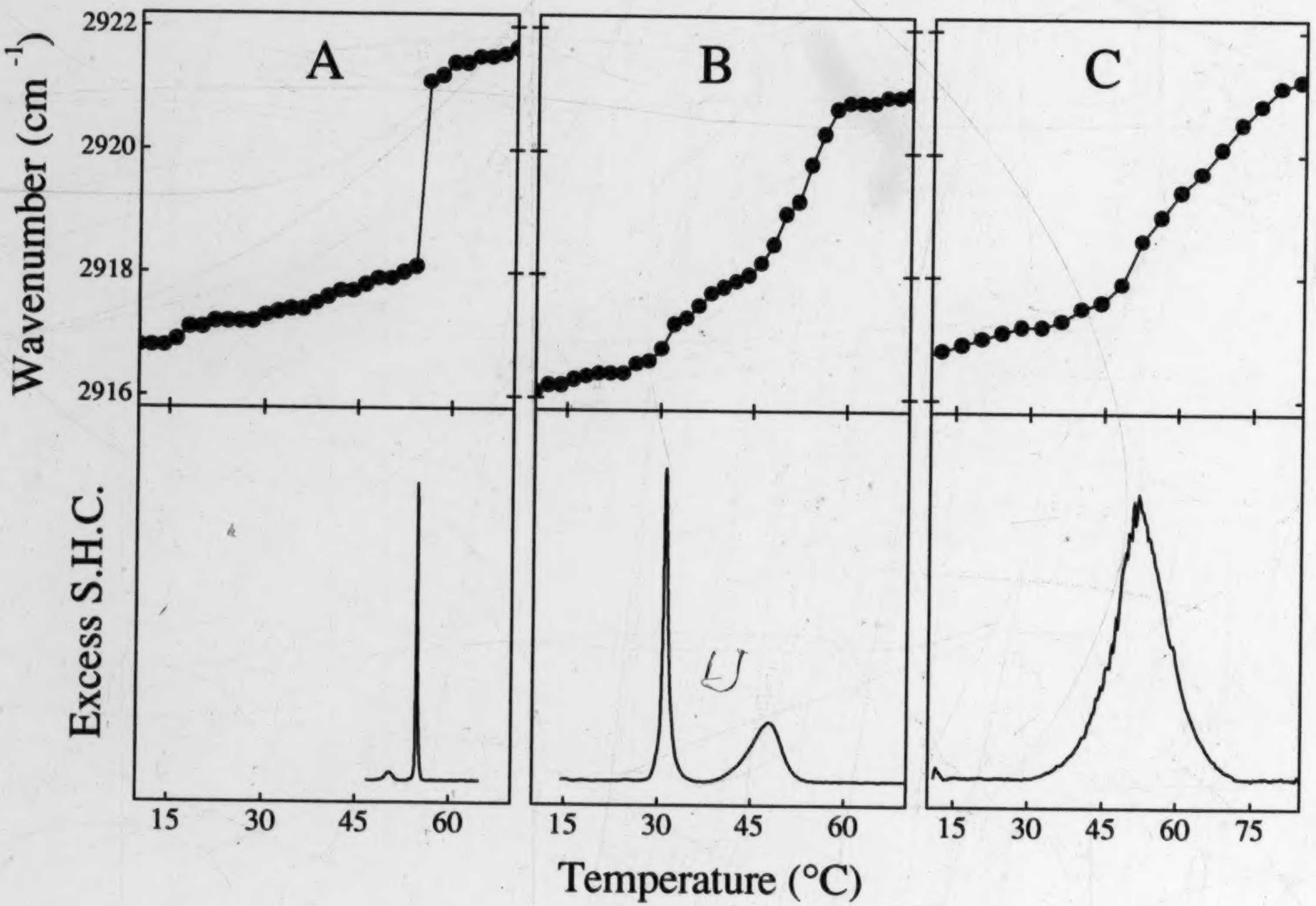
A
B
C
Wavenumber (cm -1)
2922
2920
2918
2916
Excess S.H.C.
15
30
45
60
75
Temperature (°C)

Figure IV-9. A comparison of the CD_2 asymmetric stretching absorption maxima as a function of temperature for 14:0 PC-d_{54} pure (◆), 14:0 PC-d_{54} with 25 mol% androstenol (■), and 14:0 PC-d_{54} with 25 mol% cholesterol (□). The CD_2 symmetric stretching absorption maxima are not shown but gave qualitatively similar results.

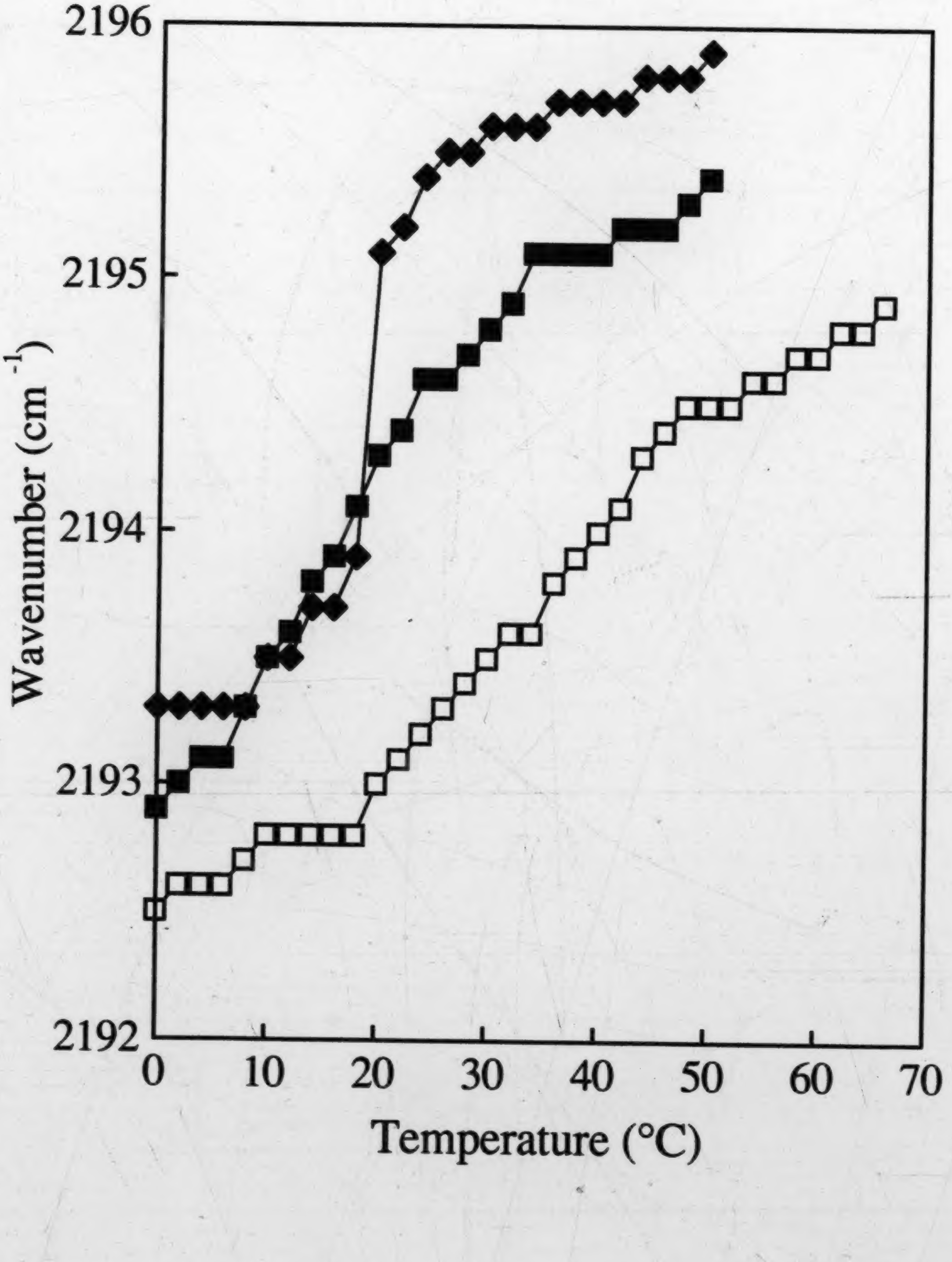
2196
2195
2194
2193
2192
Wavenumber (cm $^{-1}$)
0
10
20
30
40
50
60
70
Temperature (°C)

Figure IV-10. Comparative stacked plots of ^{31}P-NMR spectra for DMPC pure and DMPC with 25 mol% androstenol (AND) and 25 mol% cholesterol (CHOL) at temperatures both above and below their respective main transition temperatures. The temperatures for each of the spectra are as follows: below the T_m, DMPC pure (5°C), androstenol/DMPC (5°C), and cholesterol/DMPC (1°C); above the T_m, DMPC pure (40°C), androstenol/DMPC (35°C), and cholesterol/DMPC (50°C).

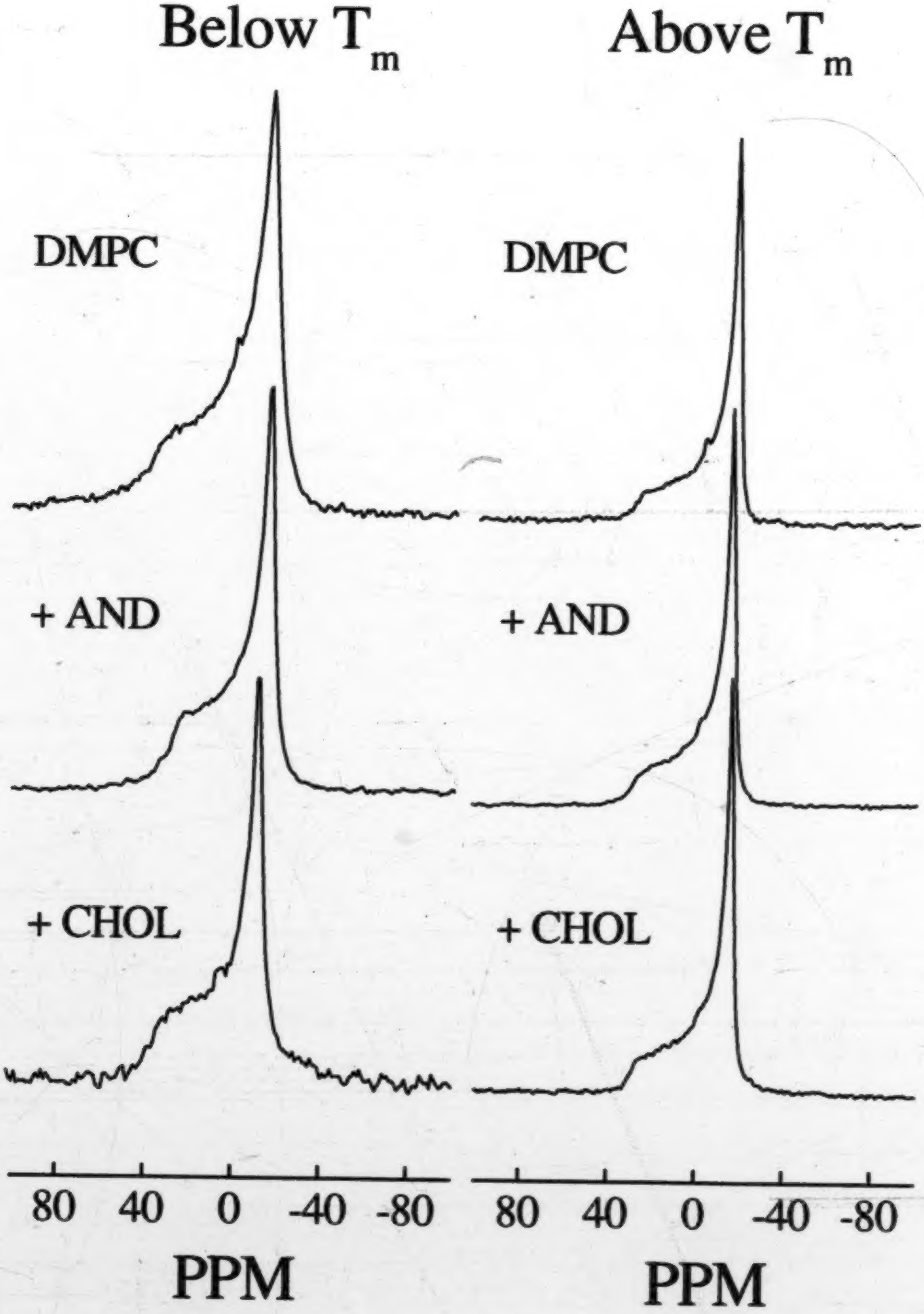
Below T_m
Above T_m
DMPC
DMPC
+ AND
+ AND
+ CHOL
+ CHOL
80
40
0
-40
-80
PPM
80
40
0
-40
-80
PPM

Figure IV-11. Comparative stacked plots of ^{31}P-NMR spectra for DSPC pure, DSPC with 35 mol% androstenol and DSPC with 35 mol% cholesterol. Plot **A**, at 5°C for all three spectra, compares spectral linewidths at temperatures below the anomalous transition of androstenol/DSPC. Plot **B**, at 35°C for all three spectra, compares spectral linewidths at temperatures between the anomalous transition and the main transition of androstenol/DSPC. Plot **C**, at 60°C for DSPC pure and androstenol/DSPC, and 70°C for cholesterol/DSPC, compares spectral linewidths at temperatures above the main transition.

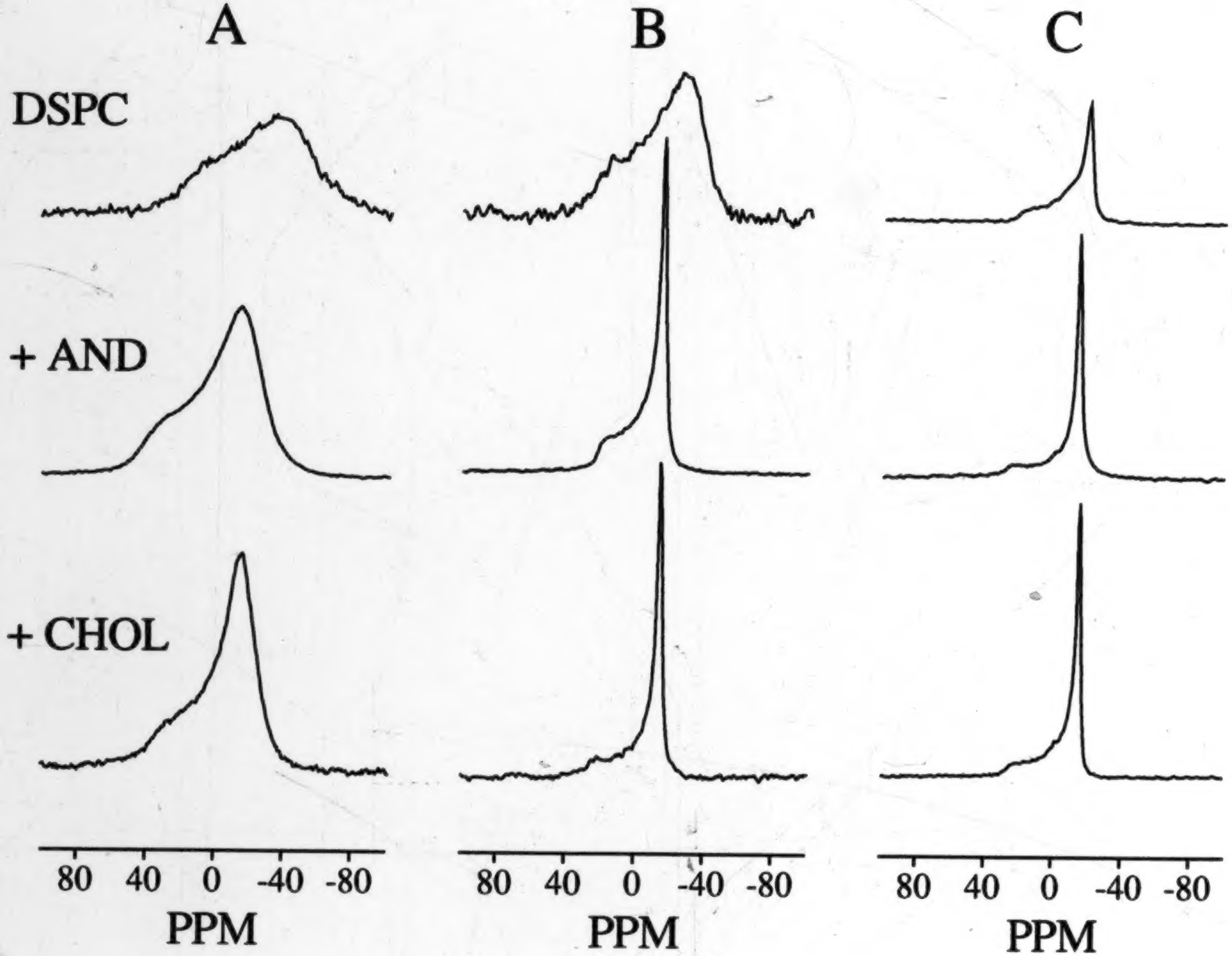
A
B
C
DSPC
+ AND
+ CHOL
80 40 0 -40 -80
PPM
80 40 0 -40 -80
PPM
80 40 0 -40 -80
PPM

Table IV-1. Chain length dependence of the androstenol/CnPC anomalous endotherm transition temperature and size.

PC	T_m (CM*)	T_m (LT**)	ΔT_m (CM-LT)	Area LT/Area CM
16:0	37.2	11.0	26.2	0.07
18:0	49.0	30.2	18.8	0.40
21:0	63.3	53.1	10.3	0.80

All temperatures in °C and scan rate corrected. All PC bilayers with 35 mol% of androstenol.

* Chain melting transition temperature

** Low temperature transition. The low temperature transition is sized relative to the chain melting transition because no stoichiometric constant is available to determine the number of moles of PC and androstenol is participating in the energetics of mixing. Hence, no direct molar enthalpic comparison is permitted between the low temperature transition and the chain melting transition for a given androstenol/PC mixture.

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CHAPTER V. DIFFERENTIAL SCANNING CALORIMETRIC STUDY OF THE EFFECT OF STEROL SIDE CHAIN LENGTH AND STRUCTURE ON DIPALMITOYLPHOSPHATIDYLCHOLINE THERMOTROPIC PHASE BEHAVIOR[5]

INTRODUCTION

Cholesterol (or a closely related sterol) is a major lipid component of the plasma membranes of most eukaryotic cells and is also found in lower concentrations in many intracellular membranes (Nes and McKean, 1977). Although cholesterol appears to have several different functions in eukaryotic cells, one of its primary roles is to modulate the physical properties of the plasma membrane phospholipid bilayer (Yeagle, 1988). Thus a large number of studies of the effects of cholesterol incorporation on the properties of phospholipid monolayers and bilayers have been carried out utilizing a wide variety of physical techniques (Demel and de Kruijff, 1976; Bittman *et al.*, 1981; Yeagle, 1985, 1988; Finean, 1990; Vist and Davis, 1990; McElhaney, 1992a). These studies have shown that cholesterol incorporation: (i) broadens and eventually eliminates the cooperative gel to liquid-crystalline phase transition of phospholipid bilayers; (ii) decreases (increases) the area per molecule of liquid-crystalline (gel) state phospholipid monolayers; (iii) increases (decreases) the orientational order of the hydrocarbon chains of liquid-crystalline (gel) phospholipid bilayers; and (iv) decreases (increases) the passive permeability of phospholipid bilayers above (below) their gel to liquid-crystalline phase transition temperatures. The presence of cholesterol in biological membranes has also been shown to modulate a number of membrane functions, presumably via its effects on the properties of the phospholipid bilayer (Dahl and Dahl, 1988; Yeagle, 1988).

A number of workers have investigated the effects of systematic variations in the structure of the cholesterol molecule on the thermotropic phase behavior, organization and passive permeability of phospholipid bilayers (Demel and de Kruijff, 1976; Yeagle, 1985, 1988; McElhaney 1992a). In general, most structural and stereochemical alterations result in some loss of the ability of the cholesterol molecule to produce its characteristic effects on phospholipid bilayers. Thus, sterols must possess an equatorially oriented C3-hydroxy group, a rigid planar fused ring system, and a flexible hydrocarbon side chain at C17 for maximum effect, while the degree of unsaturation of the ring system and the size of the alkyl side chain are of less importance. The presence of additional methyl groups on the steroid ring system, or the presence of polar functions on the alkyl side chain, also markedly reduces the effectiveness of the sterol in condensing and ordering liquid-crystalline phospholipid bilayers. Interestingly, similar structural features are required for exogenous sterols to support the maximum growth of sterol-auxotrophic mycoplasma, yeast and mammalian cells (Dahl and Dahl, 1988; McElhaney, 1992b; Bittman, 1988), confirming that one of the major roles of cholesterol in eukaryotic membranes is to regulate the physical properties of the lipid bilayer.

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[5] A version of this chapter has been published. McMullen, T.P.W., Vilchèze, C., Bittman, R. and McElhaney, R.N. (1995) *Biophys. J.* 69, 169-176.

The cholesterol analogue androstenol (5-androsten-3-β-ol), and its ring-saturated counterpart androstanol, have proven to be very useful in studies of the effect of sterols on the physical properties of phospholipid monolayers and bilayers and on the growth of sterol auxotropic cells. When androstenol, which lacks the C17 alkyl side chain, is incorporated into phospholipid bilayers, it has little effect on the physical properties of model or biological membranes. Low-sensitivity DSC studies revealed that androstenol had only small effects on the cooperativity and enthalpy of the gel to liquid-crystalline phase transition of egg PC (Ladbrooke and Chapman, 1969) or SOPC (de Kruyff *et al.*, 1972) bilayers in comparison to cholesterol. McMullen *et al.* (1994), using high-sensitivity DSC, demonstrated that the thermotropic behavior of androstenol-containing DMPC bilayers is similar to cholesterol, but with increases in PC chain length androstenol is less effective at decreasing main transition cooperativity and enthalpy due to hydrophobic mismatch-induced androstenol-PC immiscibility. Androstenol also lacks the characteristic condensing effect exhibited by cholesterol in PC monolayers (Demel *et al.*, 1972a). Moreover, ESR (Butler *et al.*, 1970; Hsia *et al.*, 1972), fluorescence polarization (Vincent and Galley, 1983) and FTIR spectroscopic (Senak *et al.*, 1992; McMullen *et al.*, 1994) studies of hydrocarbon chain order in synthetic and natural phospholipid bilayers found that androstenol was much less effective than cholesterol in reducing conformational disorder in the liquid-crystalline state. In addition, androstenol, unlike cholesterol, is unable to significantly reduce the Rb^+, glycerol, or glucose permeability of egg PC bilayers (Demel *et al.*, 1972b) or the glycerol permeability of the human erythrocyte membrane (Bruckdorfer *et al.*, 1969). Finally, androstenol is unable to support the growth of a number of cholesterol-auxotropic mycoplasma, yeast and mammalian cells (Dahl and Dahl, 1988; McElhaney, 1992b). Thus the presence of an alkyl side chain at C17 seems to be a requirement for the cholesterol molecule to exert its characteristic effects in both model and biological membranes.

Fewer studies of the effect of sterol side chain structure on sterol-phospholipid interactions have been carried out to date. ESR spectroscopic studies (Suckling and Boyd, 1976; Craig *et al.*, 1978; Suckling *et al.*, 1979) indicate that sterols containing side chains that are either shorter or longer than the isooctyl side chain of cholesterol are less effective in increasing the order of liquid-crystalline PC bilayers than is cholesterol. Moreover, the binding of the polyene antibiotics to sterols in bilayers is also dependent on sterol side chain structure (Clejan and Bittman, 1985). The rate of transbilayer movement of sterols across the membrane of growing mycoplasma cells (Clejan and Bittman, 1984), and the rate of spontaneous exchange of sterols between vesicles (Kan and Bittman, 1990; Kan *et al.* 1992) and lysophospholipid dispersions (Kan and Bittman, 1991), also depend on sterol side chain structure. However, the ability of sterols to condense sterol/DPPC monolayers is not dependent on the length or structure of the sterol side chain (Suckling *et al.*, 1979; Slotte *et al.*, 1994), nor is the degree of the reduction of the permeability of PC liposomes (Nakamura *et al.*, 1980). Similarly, while the longer and more highly branched plant sterols, such as ergosterol and sitosterol, have been reported to be as effective as cholesterol in condensing PC monolayers (Demel *et al.*, 1972a) or in ordering PC bilayers (Butler *et al.*, 1970; Vincent and Galley, 1983), other studies have found these sterols to be less effective in reducing liposomal permeability (Demel *et al.*, 1972b) or in increasing phospholipid bilayer order (Butler *et al.*, 1970; Hsia *et al.*, 1972). Clearly, additional work is required to resolve some of the apparent inconsistencies in the previous literature and to provide insight into the effect of sterol side chain structure on sterol-phospholipid interactions.

The use of high-sensitivity calorimetry has provided investigators with vital information about the physical chemistry of cholesterol/phospholipid mixtures. At cholesterol concentrations of 1-25 mol%, the DSC endotherms of cholesterol/DPPC mixtures consist of superimposed sharp and broad components, the former due to the melting of cholesterol-poor and the latter to the melting of cholesterol-rich DPPC domains (Estep *et al.*, 1978; Mabrey *et al.*, 1978; Vist and Davis, 1990; McMullen *et al.*, 1993, 1995). The sharp component exhibits a phase transition temperature and cooperativity only slightly reduced from those observed in the pure phospholipid and the enthalpy decreases to zero by approximately 20-25 mol% cholesterol. Conversely, increased levels of cholesterol decrease the broad component cooperativity significantly, while the enthalpy initially increases to a maximum at 25 mol% cholesterol, thereafter decreasing to zero by 50 mol%. Moreover, both the broad component cooperativity and transition temperature exhibit chain length-dependent behavior. Cholesterol incorporation progressively increases the transition temperature of the broad component in PCs having hydrocarbon chains of 16 or fewer carbons, while decreasing the transition temperature of PCs with hydrocarbon chains longer than 17 carbons. The broad component cooperativity also decreases more rapidly for shorter chain PCs upon cholesterol incorporation. These results were ascribed to a hydrophobic mismatch between the sterol molecule and the PC hydrocarbon chains by McMullen *et al.* (1993) (see also Mouritsen and Bloom, 1984; Chia *et al.*, 1993).

In the present work we have used high-sensitivity DSC to study the effect of a series of cholesterol analogues, varying in the length and structure of the alkyl side chain, on the thermotropic phase behavior of DPPC bilayers. The sterol analogues employed (see Figure V-1) have either terminally unbranched or terminally branched alkyl side chains varying in length from C3 to C7 and from C5 to C10, respectively. In addition, a sterol with an exo-methylene group in the side chain was examined (C-22). We find that variations in the length of the sterol side chain produce significant changes in the thermotropic phase behavior of the host DPPC bilayer relative to cholesterol. These changes can be largely explained as a result of the varying degrees of hydrophobic mismatch between the sterol and host DPPC bilayer (see also McMullen *et al.*, 1993; Chia *et al.*, 1993; Slotte *et al.*, 1994).

MATERIALS AND METHODS

The DPPC used in these experiments was purchased from Avanti Polar Lipids (Alabaster, AL). The purity was checked using TLC with a chloroform/methanol/ammonia (65:25:4) solvent system. In addition we carried out comparative high-sensitivity DSC runs with DPPC prepared in this laboratory by methods previously shown to ensure the highest purity (Lewis and McElhaney, 1985). The results were virtually identical. The sterols were synthesized using the methods described previously by Chia *et al.* (1993) and Slotte *et al.* (1994); see Figure V-1 for structures. The sterols were purified by flash chromatography on silica gel 60 (230-400 ASTM mesh) and then recrystallized from methanol. The structures were confirmed by NMR spectroscopy.

For the high-sensitivity DSC experiments, DPPC and sterol stock solutions were prepared in chloroform from which the sterol/DPPC mixtures would be prepared. The mixtures were dried under N_2 and evaporated to dryness in a vacuum overnight. Final sample preparation involved dispersion of dried sterol/DPPC mixtures in deionized water with 50 mM KCl, 1mM Na_2EDTA, and 0.05% NaN_3, heating to approximately 20 °C above the main phase transition temperature of DPPC, and then vortexing to give a multilamellar suspension. Samples were incubated in a cold room overnight before calorimetric analysis. A Hart Scientific high-sensitivity differential scanning calorimeter was used to collect the DSC thermograms. The scan rate was increased from 5 to 30 °C/hr and the amount of DPPC in each sample was increased from 1 to 9 mg with increasing sterol concentration in order to accurately monitor the broad endotherms of DPPC bilayers containing high levels of cholesterol (McMullen *et al.*, 1993).

The analysis and decomposition of the DSC endotherms was done using Microcal's (Northhampton, MA) Origin and DA-2 software. This procedure approximates each component of the endotherm as a combination of independent, two-state transitions as shown in Estep *et al.* (1978), Mabrey *et al.* (1978), McMullen *et al.* (1993), and

McMullen and McElhaney (1995). The curve broadening is expressed in terms of the van't Hoff enthalpy, which is evaluated by the equation $\Delta H=4RT^2(c_{max}/\Delta_q)$, where c_{max} is the excess specific heat capacity and Δq is the area under the curve. This protocol accurately reproduces the experimental DSC endotherms. Although other methods of estimating the temperature, enthalpy and cooperativity of the components of these DSC endotherms were employed, these yielded qualitatively similar results.

RESULTS

In the absence of sterol, unannealed DPPC bilayers exhibit two transitions, a lower temperature and lower enthalpy pretransition (conversion of the L_{β}' to the P_{β}' phase), and a higher temperature and higher enthalpy main transition (conversion of the P_{β}' to the L_{α} phase). For all of the sterols examined, incorporation of >5 mol% was sufficient to abolish the pretransition. This confirms our earlier report that the C17 side chain is not important for this particular sterol effect (McMullen *et al.*, 1994). Henceforth, our results focus on the changes observed in the main or chain-melting transition of DPPC upon the addition of different sterols.

Raw DSC thermograms for a select number of sterol concentrations are shown for DPPC bilayers containing increasing amounts of C-22 and *i*-C10 respectively, in Figures V-2a and V-2b. All sterol/DPPC mixtures exhibit two common features as a function of increasing sterol concentration: (i) a decrease in the overall enthalpy of the DPPC chain-melting endotherm; and (ii) two partially superimposed and symmetrical components within the overall asymmetric endotherm. However, at a given sterol concentration, variations in side chain length induce markedly different thermotropic behavior in the host DPPC bilayer, including dramatic differences in enthalpy, transition temperature, and cooperativity. The overall enthalpy of the DPPC chain-melting transition as a function of sterol concentration for the *n*- and *iso*-sterol series is shown in Figures V-3a and V-3b, respectively. Generally, with sterol side chains longer than 5 carbons and regardless of *n*- or *iso*- structure, the DPPC main transition enthalpy is abolished by 50 mol% sterol. In addition, the DPPC phase transition enthalpy decreases in an approximately linear manner with increases in sterol concentration. However, for the shorter chain C-22, *n*-C3 and *i*-C5 sterols, the DPPC gel to liquid-crystalline phase transition is still observable at 50 mol% sterol. The residual enthalpy of the transition at 50 mol% sterol increases with reductions in sterol side chain length. These results indicate that the effective stoichiometry of DPPC/sterol interactions depends on sterol alkyl side chain length but not structure.

Decomposition of the sharp and broad components of the DPPC main phase transition reveals the complex thermotropic behavior of these mixtures, which appears to be governed primarily by the degree of sterol-DPPC lateral phase separation. Shown in Figures V-4a and V-4b are the enthalpies of the sharp (sterol-poor phospholipid) and broad (sterol-rich phospholipid) components of the DSC endotherms as a function of sterol concentration for both sterol series. The sharp component endotherm presumably arises from a L_β to L_α phase transition and the broad component endotherm from a liquid-ordered to liquid-disordered phase transition (Vist and Davis, 1990; Huang *et al.*, 1993). In most mixtures the sharp component persists from 0 mol% to approximately 20-25 mol% sterol, with an approximately linear decline in enthalpy as a function of increasing sterol level. With C-22, *i*-C9 and *i*-C10, however, the sharp component displays significantly higher enthalpies at 15 and 20 mol% than seen with other sterols. Nevertheless, for every sterol/DPPC mixture the sharp component is abolished by approximately 30 mol% sterol. For all of the sterols examined, the enthalpy of the broad component initially increases to a maximum at approximately 20-30 mol% sterol and subsequently decreases towards 0 by 50 mol% sterol, with the exception of the shortest side chains as noted above (data not shown).

The transition temperatures of the sharp and broad components of the DPPC chain-melting endotherm are shown in Figures V-5a,b and V-6a,b, respectively. The transition temperatures of the sharp component decrease in a sterol-dependent manner. For DPPC bilayers with the *iso*-branched sterols (Figure V-5b), C-22 and *i*-C5 sterols exhibit the most significant decreases, while the rest of the *iso*-series are clustered in a temperature range only slightly reduced from the pure phospholipid. The *n*-sterol series, shown in Figure V-5a, exhibit a similarly ordered distribution in transition temperature shifts with the shortest side chain analogues exhibiting the largest decreases. A sterol-specific distribution of transition temperature shifts was also observed in the DPPC broad component upon the addition of the *n*- or *iso*-series sterols, as shown in Figures V-6a and V-6b. However, the transition temperature shifts of the broad component are significantly larger than that seen with the sharp component. Longer chain sterols induce progressive increases in the transition temperature of the broad component, while shorter chain sterols induce progressive decreases in the transition temperature of the broad component. Clearly both the sharp and broad components of the DSC endotherms of sterol/DPPC mixtures are sensitive to alterations in sterol side chain length. However, no significant difference is apparent in the thermotropic behavior of DPPC bilayers containing *iso*-branched or unbranched side chains of comparable length.

Finally, the $\Delta T_{1/2}$ of the overall DPPC endotherm also varies depending on sterol side chain structure (Figures V-7a and V-7b). DPPC bilayers with shorter chain sterols generally exhibit significantly increased endotherm $\Delta T_{1/2}$ values (lower cooperativity) as compared to longer chain sterols at comparable levels of sterol incorporation. By 20 mol% sterol the shorter chain sterols exhibit $\Delta T_{1/2}$ values twice as large as the longer side chain sterols. This holds true for both the *n*- and *iso*-series of sterols. While this general trend is clearly observable in DPPC bilayers containing lower sterol levels (<25 mol%), increases in sterol incorporation toward 40-50 mol% obscure this relationship because of the increased immiscibility of the shorter chain sterols in the gel-state DPPC bilayers (see McMullen *et al.*, 1994).

DISCUSSION

Our results indicate that even small changes in the length of the C17 side chain of cholesterol will alter the thermotropic phase behavior of the host DPPC bilayer. Each sterol induces significantly different shifts in the transition temperature, enthalpy and cooperativity of the sharp and broad components of sterol/DPPC mixtures. However, our observations, as well as those of Chia *et al.* (1993), indicate that differences in the structure (*n*- vs *iso*-) of the sterol side chain do not significantly alter the thermotropic behavior of DPPC bilayers. Thus the observed results conform well to that predicted by the hydrophobic mismatch effect as outlined for sterol/PC systems by McMullen *et al.* (1993, 1994) and by Chia *et al.* (1993).

In all of the sterol/DPPC mixtures the sharp, sterol-poor, phospholipid chain-melting endotherm exhibited only relatively small changes in transition temperature and cooperativity. However the broad, sterol-rich, phospholipid chain-melting endotherm exhibited dramatic and progressive differences in transition temperature and cooperativity whose direction and magnitude depended on the length but not the structure of the alkyl chain of the sterol. The differences in the transition temperature and cooperativity of the broad component of the different sterol/DPPC mixtures seem to arise primarily from varying degrees of hydrophobic mismatch between the sterol and the host DPPC bilayer. The hydrophobic region of the bilayer is defined as the region between the *sn*-1 carbonyl groups of DPPC molecules in opposing monolayers. At the gel to liquid-crystalline phase transition, the hydrophobic thickness of the PC bilayers decreases by approximately one-third. The presence of another amphiphile in the bilayer which does not undergo a similar change in hydrophobic thickness will differentially affect gel and liquid-crystalline bilayer stability unless its length is approximately equal to the mean thickness of the host bilayer (i.e., the sterol has a hydrophobic length midway between that of the gel and liquid-crystalline DPPC phases). Thus sterols with lengths greater than the mean thickness of the host PC bilayer will preferentially stabilize the gel phase of a PC bilayer (increase transition temperature), while short side chain sterols will destabilize the gel state (decrease transition temperature) (Mouritsen and Bloom, 1984). We previously demonstrated that the effective length of the cholesterol molecule is approximately 17.5 Å, close to the mean hydrophobic length of di-17:0 PC (McMullen *et al.*, 1993). The mean hydrophobic thickness of one of the DPPC monolayers is approximately 16.5 Å; thus we predict and observe a slight increase in the transition temperature of the cholesterol-rich broad component of the DPPC endotherm. With changes in the sterol side chain length from approximately 19.5 Å for *i*-C10 to 13.0 Å for C-22 or *i*-C5, we see progressive increases and decreases, respectively, in the transition temperature of the broad component of the DPPC chain-melting endotherm. For intermediate length sterols we observe a general progression of transition temperatures between the two extremes, C-22 or *i*-C5 and *i*-C10. These results complement the CH_2 wagging progression analysis of DPPC bilayers containing a similar series sterols by Chia *et al.* (1993). These workers determined that sterols with shorter side chains (<C6) induce a significantly higher degree of conformational disorder in the DPPC hydrocarbon chains than cholesterol for a given temperature. Conversely, liquid-crystalline DPPC bilayers containing longer chain sterols (>C8) exhibit a greater degree of conformational order relative to cholesterol. Moreover, CH_2 wagging intensity profiles as a function of temperature indicate that the chain melting of DPPC bilayers containing shorter chain sterols occurs at significantly lower temperatures than that seen for cholesterol and vice versa. These spectroscopic results clearly corroborate our DSC results and the hydrophobic mismatch model of sterol/PC interactions.

With most of the sterols studied here, the decreases in the DPPC chain-melting transition enthalpy as a function of increasing sterol concentration are similar, with an approximately linear decrease to zero by 50 mol% sterol. Thus the effective stoichiometry of sterol/DPPC interactions is not dependent on the length or structure of the alkyl side chain of the sterol. However, for the shorter chain sterols, a residual DPPC chain-melting phase transition can be detected at sterol concentrations above 50 mol%. We suggest that as the degree of hydrophobic mismatch between these sterols and the host DPPC bilayer increases, these sterols may no longer be completely miscible in the gel-state bilayer. As a result the effective stoichiometry of sterol/DPPC interactions is altered such that, on average, each DPPC molecule is in contact with fewer sterol molecules. This is seen with androstenol in DPPC and DSPC bilayers, where a significant chain-melting transition is apparent even at androstenol levels of 50 mol% (McMullen *et al.*, 1994). Also, the appearance of a new, low temperature endotherm was noted in these systems at androstenol concentrations as low as 5 mol%. The low temperature endotherm was shown to be the result of temperature-induced dissolution of phase-separated androstenol in these gel state PC bilayers. Clearly with C-22, and to a lesser degree *i*-C3 and *i*-C5, the chain-melting transition observable at 50 mol% sterol is indicative of partial gel-state sterol immiscibility due to sterol/DPPC hydrophobic mismatch. However, the presence of an alkyl side chain, even as short as three carbons, is apparently sufficient to prevent a lateral segregation of sterol molecules in gel-state DPPC bilayers as is observed with androstenol.

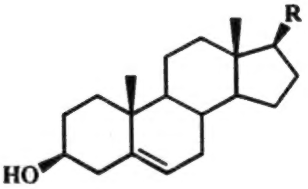
The hydrophobic mismatch effect also accounts for the variations in the main transition cooperativity of the various sterol/DPPC mixtures. Due to the significant disordering of the DPPC gel-state bilayer by the incorporation of sterols such as C-22, *i*-C5 and *n*-C3, the temperature required to induce increased *gauche* conformer formation decreases. Decreased conformational order in short chain sterol-containing DPPC bilayers compared to cholesterol-containing DPPC bilayers was documented by Chia *et al.* (1993). The result is a broader, lower temperature endotherm as seen by DSC. The converse is true with DPPC bilayers with longer chain sterols. Increased steric restriction of the PC hydrocarbon chain by the sterol side chain, although to a lesser degree than that induced by the sterol ring system, significantly increases the conformational order in the DPPC hydrocarbon chains compared to a sterol lacking a side chain. The result is a more cooperative phase transition at higher temperatures. Note that under conditions of gel-state immiscibility, such as with C-22, the decrease in the cooperativity of the phase transition as a function of increasing sterol concentration is significantly reduced. This is due to the segregation of short-chain sterols into phase-separated sterol-rich domains, thus lowering the number of effective sterol/DPPC contacts and reducing the influence of the sterol on DPPC thermotropic behavior.

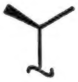
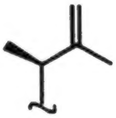
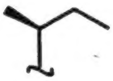
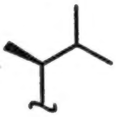
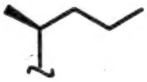
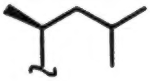
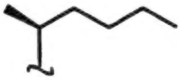
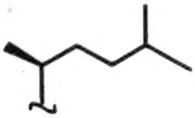
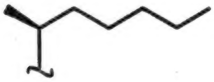
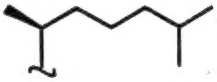
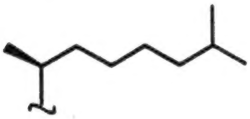
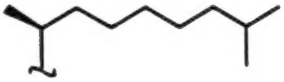
The complex and sometimes conflicting results observed in prior calorimetric and spectroscopic studies of cholesterol side chain analogues in phospholipid bilayers may be related in part to the complex thermotropic behavior of these systems. This study, as well as those by Chia *et al.* (1993) and Slotte *et al.* (1994), clearly demonstrate that the thermotropic behavior and organization of sterol-containing DPPC mixtures depend on sterol side chain length. Depending on the sterol and the host phospholipid bilayer, the degree of hydrophobic mismatch may lower or raise the temperature required for the gel to liquid-crystalline phase transition. Moreover, the phospholipid conformational order as a function of temperature would be dramatically different than that observed for cholesterol-containing bilayers. Thus the conflicting observations of studies examining the physical properties of various sterols (see Introduction) on phospholipid bilayers may be due in part to different sterol-induced shifts in the gel to liquid-crystalline phase transition of the host phospholipid bilayer as well as differences in sterol gel-state miscibility. The results of a parallel calorimetric study of these same side chain analogues in SOPC bilayers support this conclusion. We believe that comparative studies of the effect of sterols on phospholipid thermotropic phase behavior, hydrocarbon chain conformational order, and permeability should be carried out on the same host phospholipid bilayer, and at comparable reduced temperature.

Finally, this study demonstrates, in conjunction with the studies of McMullen *et al.* (1993, 1994) and the spectroscopic data of Senak *et al.* (1992) and Chia *et al.* (1993), that the complex sterol/DPPC thermograms obtained by DSC represent the behavior of superimposed but independent symmetric endotherms. This determination was made possible by the sterol-specific differential temperature shifts exhibited by the sharp and broad components of the DPPC endotherm. Moreover, the temperature and width of the broad component of the DSC endotherm correlate well with the temperature-dependent changes observed in acyl chain conformational order of cholesterol-containing DPPC bilayers as observed by Chia *et al.*, (1993) using FTIR spectroscopy. Thus the DSC endotherm curves accurately represent the chain-melting of both the cholesterol-rich and cholesterol-poor DPPC domains, and the decomposition of the overall endotherm enables us to determine the relative proportions of each of the domains.

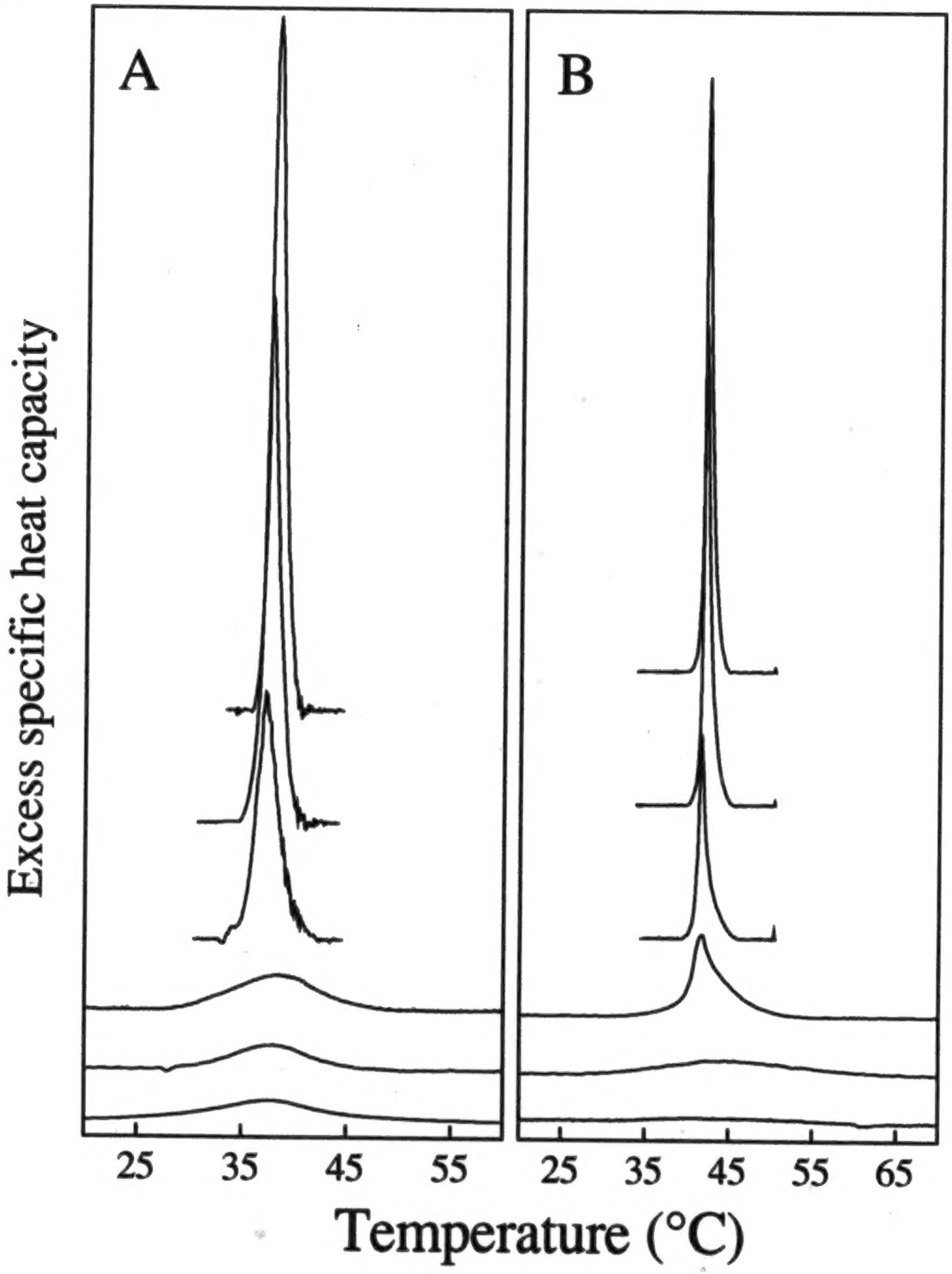
These results confirm that the evolution of the structure of cholesterol is based in part on regulation of the physical properties of the phospholipid bilayer. The complexities of the thermotropic behavior and organization of sterol/PC mixtures are only now becoming apparent with application of high-sensitivity DSC and various spectroscopic techniques to these systems. Further examination of sterol/phospholipid mixtures in which both components are systematically varied may reveal additional features of these systems not yet identified.

Figure V-1. Sterol side chain structures and their abbreviations as used in this paper.

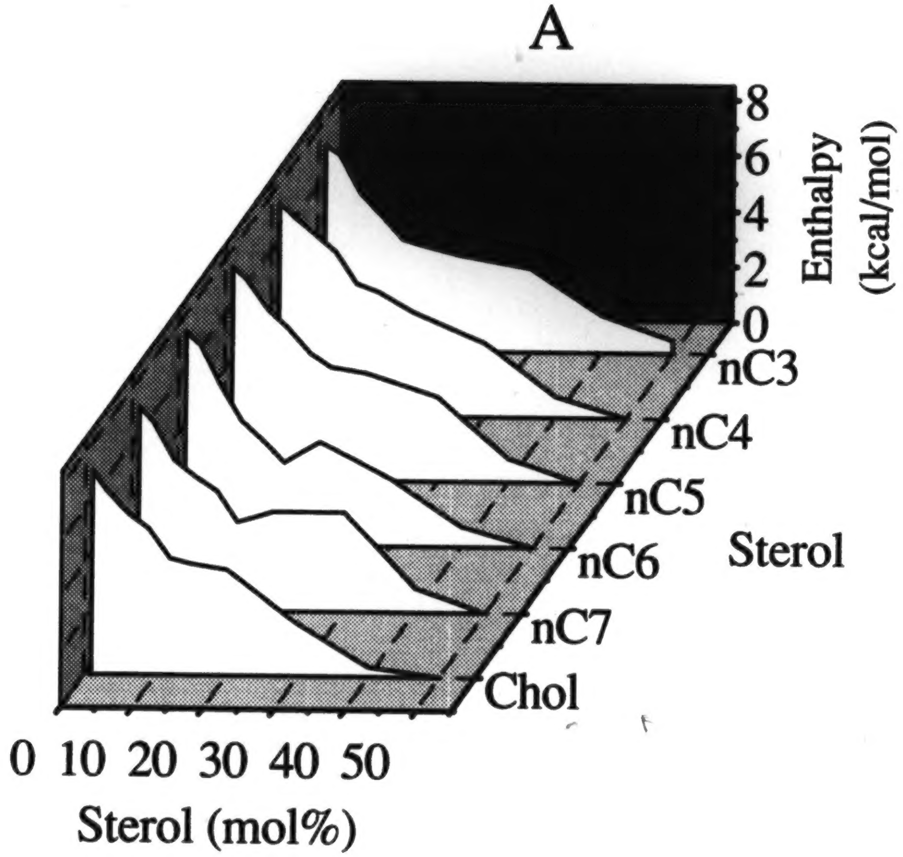


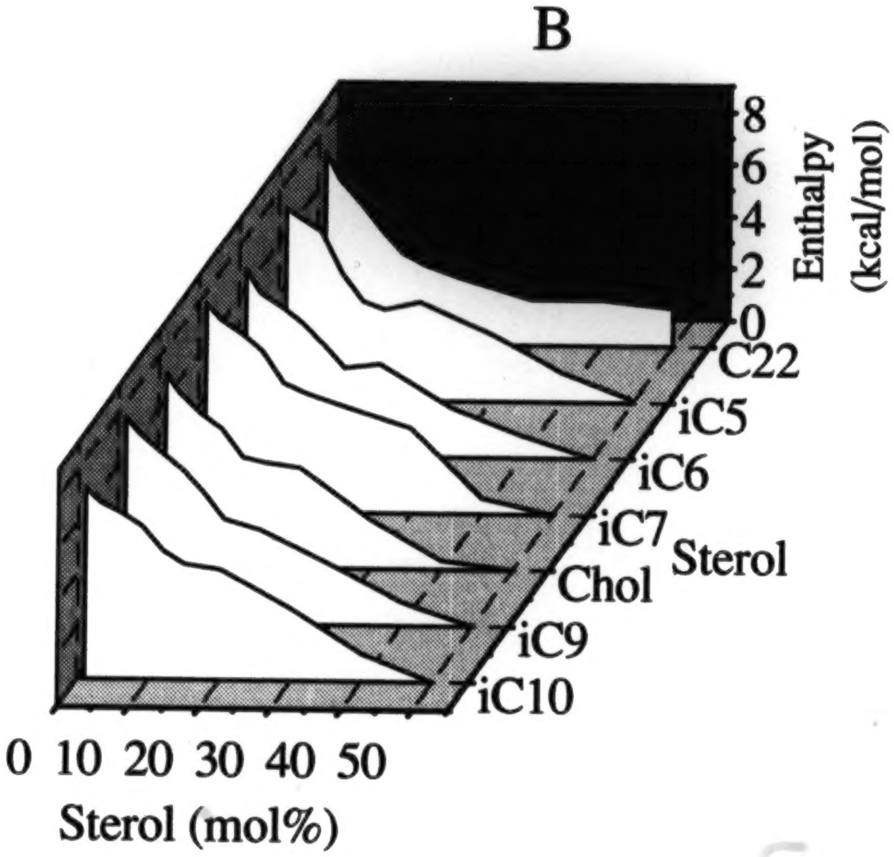
COMPOUND	CODE NAME	COMPOUND	CODE NAME
R =		R =	
	*n*-C3		C-22
	*n*-C4		*i*-C5
	*n*-C5		*i*-C6
	*n*-C6		*i*-C7
	*n*-C7		cholesterol
			*i*-C9
			*i*-C10

Figures V-2a and V-2b. Representative plots of DPPC bilayer endotherms as a function of increasing C-22 (A) and *i*-C10 (B) sterol concentration. Sterol concentrations, shown in descending order, are 3, 6, 9, 12, 20, and 40 mol%. Sterol concentrations done but not shown are 0, 15, 25, and 30 mol%.

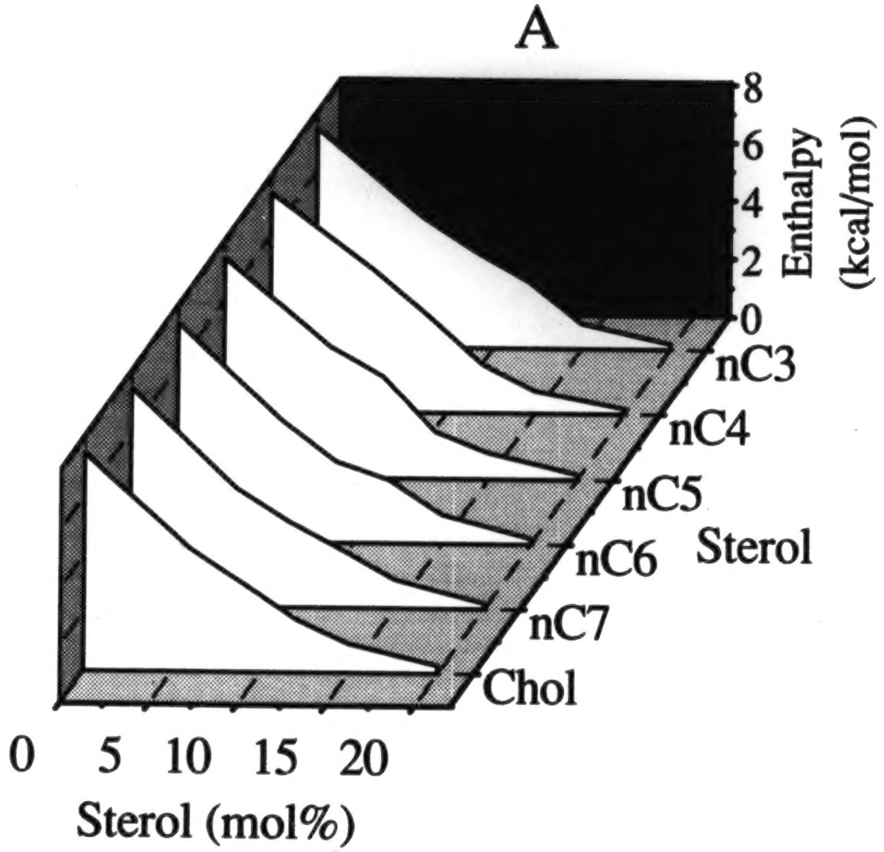

A
B
Excess specific heat capacity
25
35
45
55
25
35
45
55
65
Temperature (°C)

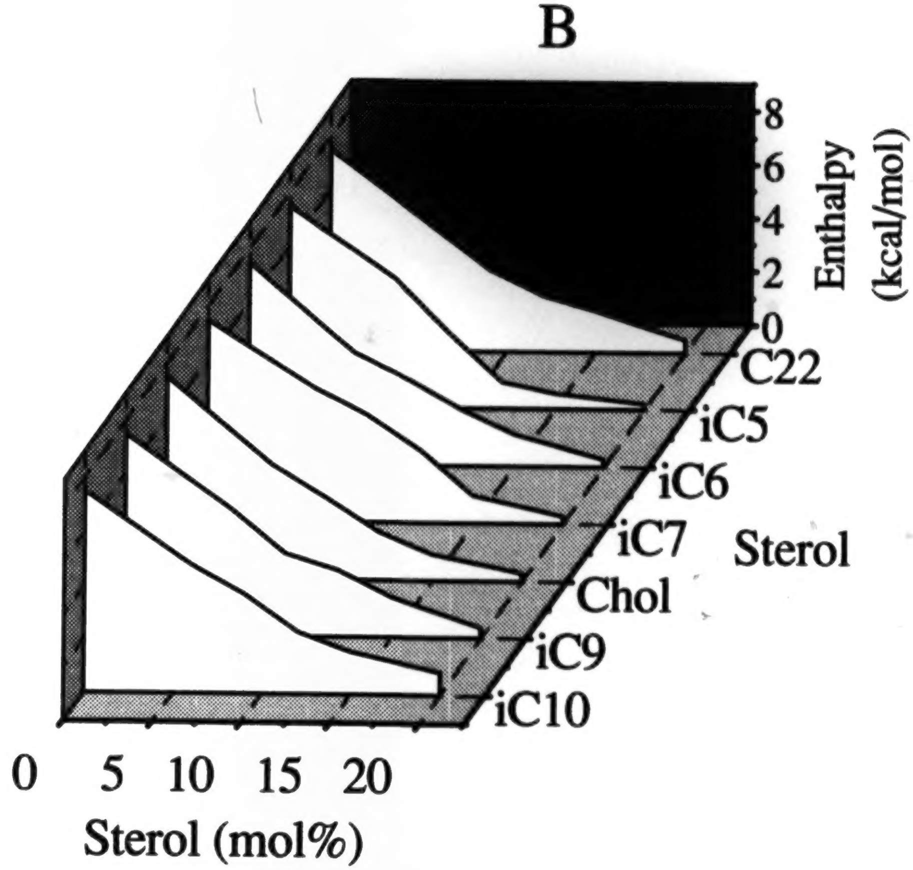
Figures V-3a and V-3b. Three dimensional plot of the effect of increasing sterol concentrations on the DPPC main transition overall enthalpy for the (A) *n*- and (B) *iso*-series of sterols. Sterols and their respective concentrations and are shown on the figure.


A
8
6
4
2
0
Enthalpy (kcal/mol)
nC3
nC4
nC5
nC6
nC7
Chol
Sterol
0 10 20 30 40 50
Sterol (mol%)

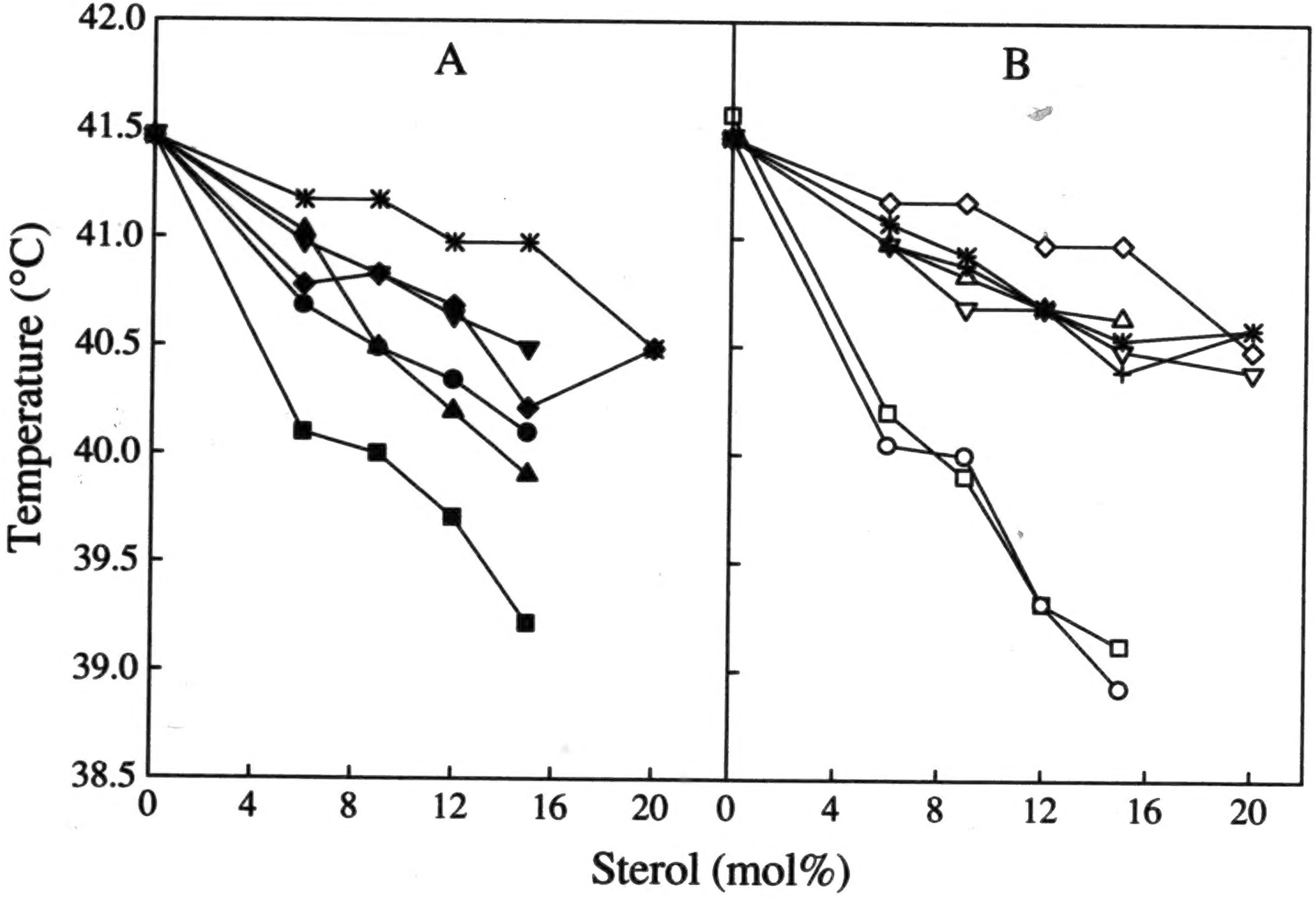

B
8
6
4
2
0
Enthalpy (kcal/mol)
C22
iC5
iC6
iC7
Chol
iC9
iC10
Sterol
0
10
20
30
40
50
Sterol (mol%)

Figures V-4a and V-4b. Sharp component enthalpy as a function of increasing sterol concentration for (A) the *n*- and (B) *iso*-series sterols. Sterols and their respective concentrations are shown on the figure.

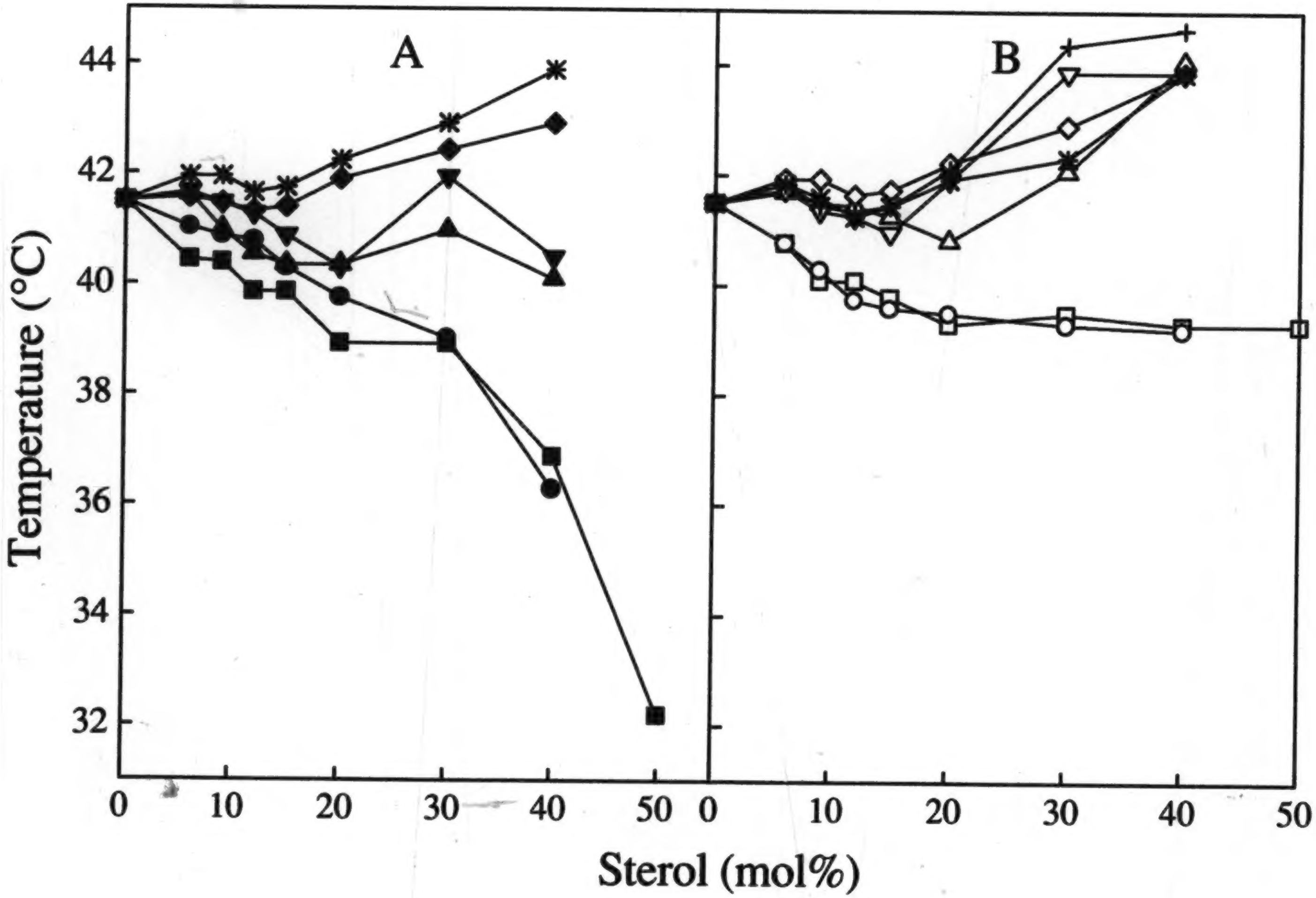

A
8
6
4
2
0
Enthalpy (kcal/mol)
nC3
nC4
nC5
nC6
nC7
Chol
Sterol
0
5
10
15
20
Sterol (mol%)


B
8
6
4
2
0
Enthalpy (kcal/mol)
C22
iC5
iC6
iC7
Chol
iC9
iC10
Sterol
0
5
10
15
20
Sterol (mol%)

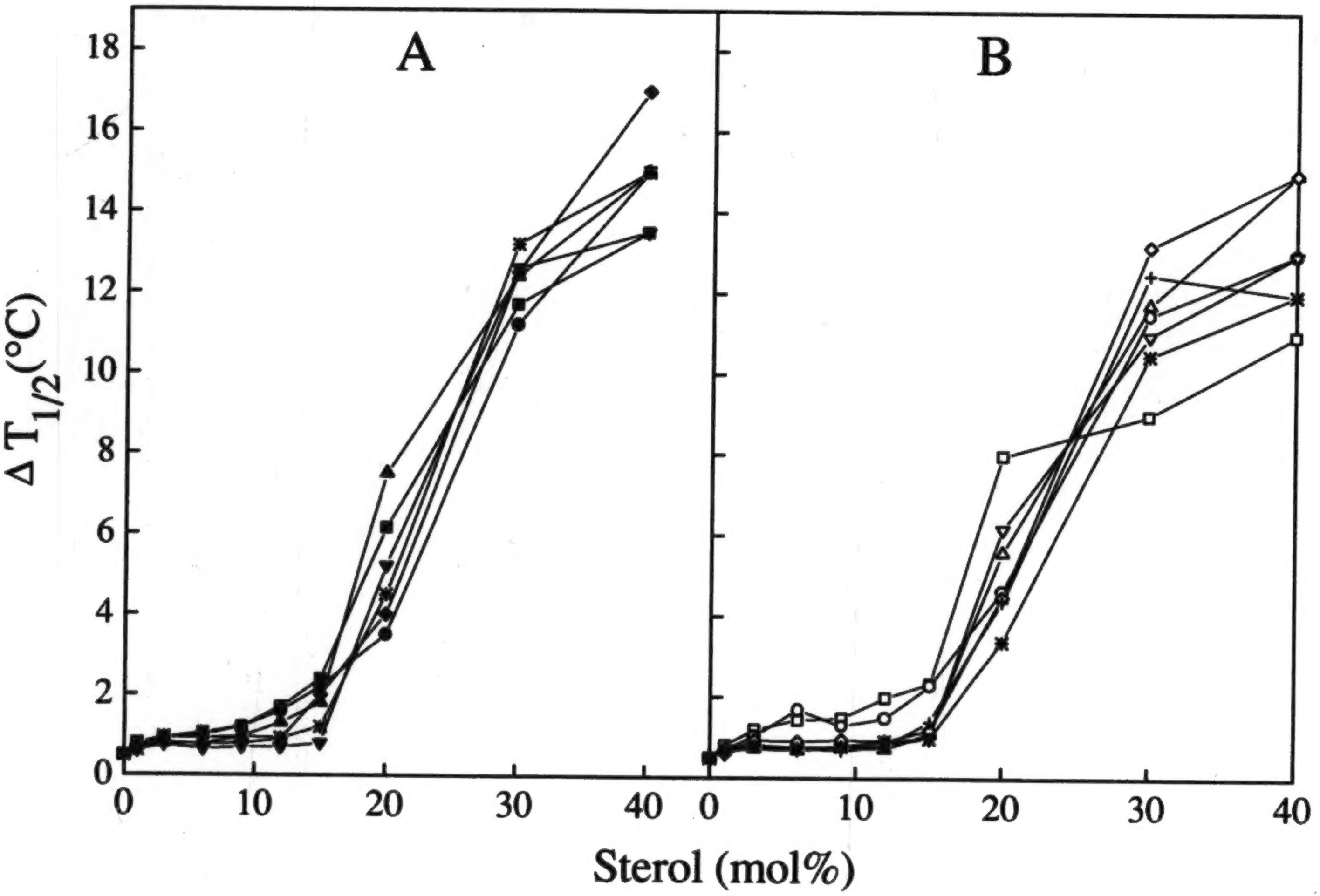
Figures V-5a and V-5b. Sharp component transition temperature as a function of increasing sterol concentration: (**A**) *n*-C3 (■); *n*-C4 (●); *n*-C5 (▲); *n*-C6 (▼); *n*-C7 (◆); Cholesterol (*), (**B**) C-22 (❑); *i*-C5 (○); *i*-C6 (Δ); *i*-C7(∇); Chol (◊); *i*-C9 (+); *i*-C10 (*).


A
B
Temperature (°C)
42.0
41.5
41.0
40.5
40.0
39.5
39.0
38.5
0
4
8
12
16
20
Sterol (mol%)

Figures V-6a and V-6b. Broad component transition temperature as a function of increasing sterol concentration: (**A**) *n*-C3 (■); *n*-C4 (●); *n*-C5 (▲); *n*-C6 (▼); *n*-C7 (◆); Cholesterol (*), (**B**) C-22 (□); *i*-C5 (○); *i*-C6 (Δ); *i*-C7(∇); Chol (◊); *i*-C9 (+); *i*-C10 (*).


A
B
Temperature (°C)
44
42
40
38
36
34
32
0
10
20
30
40
50
0
10
20
30
40
50
Sterol (mol%)

Figures V-7a and V-7b. Overall DPPC endotherm $\Delta T_{1/2}$ as a function of increasing sterol concentration: (**A**) *n*-C3 (■); *n*-C4 (●); *n*-C5 (▲); *n*-C6 (▼); *n*-C7 (◆); Cholesterol (*), (**B**) C-22 (❑); *i*-C5 (○); *i*-C6 (Δ); *i*-C7(∇); Chol (◊); *i*-C9 (+); *i*-C10 (*).


A
B
ΔT1/2(°C)
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
0
10
20
30
40
Sterol (mol%)

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CHAPTER VI. THE EFFECT OF SIDE CHAIN ANALOGUES OF CHOLESTEROL ON THE THERMOTROPIC PHASE BEHAVIOR OF 1-STEAROYL-2-OLEOYL-PHOSPHATIDYLCHOLINE BILAYERS: A DIFFERENTIAL SCANNING CALORIMETRIC STUDY[6]

INTRODUCTION

The occurrence of high concentrations of cholesterol in the plasma membrane of higher organisms has prompted numerous investigations into its role in the structure and function of cell membranes (Dahl and Dahl, 1988; Yeagle, 1988). One of the primary roles of cholesterol in eukaryotic cells is to modulate the physical properties of the plasma membrane phospholipid bilayer (Yeagle, 1988). Thus a large number of studies of the effects of cholesterol incorporation on the properties of phospholipid monolayers and bilayers have been carried out utilizing a wide variety of physical techniques (Demel and De Kruijff, 1976; Yeagle, 1985, 1988; Finean, 1990; Vist and Davis, 1990; McElhaney, 1992a). These studies have shown that cholesterol decreases the area per molecule of liquid-crystalline phospholipid monolayers. Moreover, cholesterol increases the orientational order of the hydrocarbon chains of liquid-crystalline phospholipid bilayers and eventually eliminates their cooperative gel to liquid-crystalline phase transition. Lastly, cholesterol incorporation also decreases the passive permeability of phospholipid bilayers above their gel to liquid-crystalline phase transition temperatures.

A number of workers have investigated the effects of systematic variations in the structure of the cholesterol molecule on the thermotropic phase behavior, organization and passive permeability of phospholipid bilayers (Yeagle, 1985, 1988; Demel and de Kruijff, 1976; McElhaney, 1992a). For example, 5-androsten-3β-ol, an analogue of cholesterol that lacks the C17 isooctyl side chain, is incapable of condensing PC monolayers (Demel *et al.*, 1972; Slotte *et al.*, 1994) and is less effective than cholesterol in reducing the solute permeability of egg PC (Demel *et al.*, 1972) and erythrocyte bilayers (Demel *et al.*, 1972; Bruckdorfer *et al.*, 1969; Nakamura *et al.*, 1980), or in decreasing the conformational disorder of PC fatty acyl chains (Butler *et al.*, 1970; Hsia *et al.*, 1972; Vincent and Gallay, 1983; Senak *et al.*, 1992; Chia and Mendelsohn, 1992; Chia *et al.*, 1993; McMullen *et al.*, 1994). Androstenol is also less effective than cholesterol in decreasing the main phase transition enthalpy and cooperativity of egg PC (Ladbrooke and Chapman, 1969), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) (De Kruijff *et al.*, 1972), or saturated PCs with chain lengths longer than 14 carbons (McMullen *et al.*, 1994). Moreover, sterols that have either a shorter or longer side chain than cholesterol are not as effective as cholesterol in increasing the hydrocarbon chain order of liquid-crystalline PC bilayers (Suckling and Boyd 1976; Craig *et al.*, 1978; Suckling *et al.*, 1979). The binding of polyene antibiotics to sterols in bilayers is also dependent on sterol side chain structure (Clejan and Bittman, 1985) as is the rate of transbilayer movement of sterols across the membrane of growing mycoplasma cells (Clejan and Bittman, 1984) and the rate of spontaneous exchange of sterols between vesicles (Kan and Bittman, 1990; Kan *et al.*, 1992) and lysophospholipid dispersions (Kan and Bittman, 1991). Thus most structural and stereochemical alterations result in some loss of the ability of the cholesterol molecule to produce its characteristic effects on phospholipid bilayers. The same is true for the ability of exogenous sterols to support the maximum growth of sterol-auxotrophic mycoplasma, yeast and mammalian cells (Dahl and Dahl, 1988; McElhaney, 1992b; Bittman, 1988), confirming that one of the major roles of cholesterol in eukaryotic membranes is to regulate the physical properties of the lipid bilayer.

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[6] A version of this chapter has been published. Vilchèze, C., McMullen, T.P.W., McElhaney, R.N. and Bittman, R. (1996) *Biochim. Biophys. Acta* 1279: 235-242.

The vast majority of previous DSC studies of cholesterol-phospholipid interactions have used PC's bearing two identical saturated fatty acyl chains. However, except in lung surfactant and nerve endings where high levels of DPPC are found, naturally occurring PC's rarely contain identical fatty acyl chains (O'Brien and Gerson, 1974). Instead, PC's that contain a saturated chain at the *sn*-1 position and an unsaturated chain at the *sn*-2 position are most abundant in biological membranes (Kuksis, 1978). Prior low-sensitivity DSC studies of the effect of cholesterol on the thermotropic behavior of mixed-chain PCs revealed that cholesterol interacts differentially with *sn*-1 and *sn*-2 positional isomers (Davis and Keough, 1983; Kariel *et al.*, 1991; Hernandez-

Borrell and Keough, 1993). In addition, PC bilayers with various levels of unsaturation exhibit significantly different thermotropic responses to the incorporation of cholesterol (Kariel *et al.*, 1991). In order to provide additional insights into the effects of sterol alkyl side chain length and branching on sterol interactions with mixed-chain saturated-unsaturated PC bilayers, we have undertaken the present high-sensitivity differential scanning calorimetric study of sterol/SOPC mixtures (see Figure VI-1 for the structures of the cholesterol analogues used). We find that variations in the length, but not the structure, of the sterol side chain produce significant changes in the thermotropic phase behavior of the host SOPC bilayer. Although the effects of these cholesterol side chain analogues on the thermotropic behavior of SOPC and DPPC (McMullen *et al.*, 1995) bilayers are qualitatively similar, significant quantitative differences are also observed. We ascribe these differences in the thermotropic behavior of sterol/SOPC and sterol/DPPC mixtures primarily to differences in the degree of hydrophobic mismatch between the sterol and host bilayer and secondarily to differences in the interaction of these sterols with saturated and unsaturated *sn-2* hydrocarbon chains in the gel state (McMullen *et al.*, 1993; Huang, 1977; Li *et al.*, 1994; Wang *et al.*, 1995)

MATERIALS AND METHODS

The SOPC used in these experiments was purchased from Avanti Polar Lipids (Alabaster, AL). The purity was checked using thin layer chromatography with a chloroform/methanol/ammonia (65:25:4) solvent system. Both moderately and heavily loaded plates showed only a single spot at the expected position upon visualization. The cholesterol side chain analogues used in this study were synthesized as described previously (Chia *et al.*, 1993). These cholesterol analogues were purified by flash chromatography on silica gel 60 (230-400 ASTM mesh) and then recrystallized from methanol. Their structures were confirmed by NMR spectroscopy (Chia *et al.*, 1993).

The sterol/SOPC binary mixtures were prepared from SOPC and sterol stock solutions in chloroform. The sterol/SOPC mixtures were dried under N_2 and evaporated to dryness in a vacuum overnight. The dried lipid mixture was then suspended in deionized water containing 50 mM KCl, 1 mM Na_2EDTA, and 0.05% NaN_3 and heated approximately 20 °C above the phase transition temperature with vortexing to give a multilamellar suspension. These aqueous dispersions were stored overnight under N_2 at 4°C. A Hart Scientific high-sensitivity differential scanning calorimeter (Pleasant Grove, UT) was used to collect the DSC thermograms. Samples were incubated in the calorimeter at -10°C for at least 60 minutes prior to the start of the DSC heating scans to ensure an initial state of equilibrium. Each sample was scanned at least three times. The DSC scan rates were increased with increasing sterol concentration, from 5 to 30 °C/h, and the amount of SOPC in each sample was increased from 2 to 10 mg. This experimental protocol ensured that the broad, low-enthalpy endotherms observed at high sterol concentrations are accurately recorded (McMullen *et al.*, 1993).

The analysis and decomposition of the DSC endotherms was done using Microcal (Northhampton, MA) Origin and DA-2 software. This procedure approximates each component of the endotherm as a combination of independent, two-state transitions as shown previously (McMullen *et al.*, 1993; Estep *et al.*, 1978; Mabrey *et al.*, 1978; McMullen and McElhaney, 1995). The curve broadening is expressed in terms of the van't Hoff enthalpy, which is evaluated by the equation $\Delta H=4RT^2(c_{max}/\Delta_q)$, where c_{max} is the excess specific heat capacity and Δ_q is the area under the curve. This protocol accurately reproduces our experimental DSC endotherms. Although other methods of estimating the temperature, enthalpy and cooperativity of the components of these DSC endotherms were also employed, these analyses yielded qualitatively similar results.

RESULTS

High-sensitivity DSC thermograms of SOPC in the absence of sterol are presented at the top of Figure VI-2; only the bottom half of these very sharp endotherms could be accommodated in this figure without a drastic reduction of the excess specific heat scale. Pure SOPC exhibits a single, fully reversible gel to liquid-crystalline phase transition over the temperature range examined. The transition temperature is 6.0 °C, the transition enthalpy 5.9 kcal/mol, and the apparent $\Delta T_{1/2}$ (a measure of the cooperativity of this chain-melting phase transition) is about 0.75 °C. The transition temperature and enthalpy values almost exactly match the average values for SOPC reported in previous low- and high-sensitivity DSC studies but the $\Delta T_{1/2}$ values we report here are much smaller (the SOPC phase transition is considerably more cooperative). These sharp DSC endotherms confirm that the sample of SOPC used in these experiments is of high purity.

Shown in Figures VI-2a and VI-2b are DSC endotherms of the main phase transition of SOPC bilayers as a function of increasing sterol concentration for the branched short side chain sterol C-22 and branched long side chain sterol *i*-C10, respectively. It is clear that in both mixtures that the addition of cholesterol analogues dramatically alters the enthalpy, transition temperature, and cooperativity of the overall SOPC gel to liquid-crystalline phase transition. Moreover, the overall endotherm for each sterol/SOPC mixture is clearly a composite of two overlapping components. This multicomponent melting behavior is exhibited by all of the sterol/SOPC mixtures examined. However, the shifts in enthalpy, transition temperature, and cooperativity of the overall endotherm vary significantly depending on the sterol. The detailed data obtained with each sterol are described in the following paragraphs.

The overall enthalpy of the SOPC main phase transition as a function of increasing sterol concentration is shown in Figure VI-3a for the *n*-series and in Figure VI-3b for the *iso*-series of cholesterol side chain analogues. For the *n*-series sterols, the overall chain-melting phase transition enthalpy of SOPC decreases progressively towards zero by 50 mol% sterol, with the exception of *n*-C3, where residual enthalpy persists at high sterol levels. (We did not study *n*-series sterols with side chains longer than that of cholesterol.) We observed a similar progressive decrease in enthalpy for SOPC bilayers containing the *iso*-series sterols; however, the overall chain-melting transition of SOPC persisted at sterol levels of 50 mol% with the shorter side chain sterols C-22 and *i*-C5 and with the longer side chain sterols and *i*-C9 and *i*-C10. This effect is much more pronounced for the shorter chain sterols, as indicated by the larger residual enthalpy at 50 mol% sterol. The effect of cholesterol analogues containing either linear or branched side chains on the overall enthalpy of the gel to liquid-crystalline phase transition of the host SOPC bilayer were similar at comparable sterol concentrations.

Since the chain-melting phase transition of SOPC clearly consists of both a sharp (sterol-poor) and a broad (sterol-rich) melting component at lower sterol concentrations, we decomposed the overall endotherm to detail the sterol-dependent behavior of each component (Davis and Keough, 1983; McMullen *et al.*, 1993; Estep *et al.*, 1978; Mabrey *et al.*, 1978; McMullen and McElhaney, 1995). The variation in the enthalpy of the sharp component with sterol concentration for the *n*-series of cholesterol analogues is shown in Figure VI-4a. With the exception of *n*-C7, the enthalpy of the sharp component of each mixture decreases linearly toward zero by approximately 20 mol% sterol. The *iso*-series sterols, shown in Figure VI-4b, generally exhibit a similar behavior; however, the enthalpy of the sharp component of SOPC bilayers containing the shortest side chain sterol (C-22) or the longer side chain sterols (*i*-C7, *i*-C9 and *i*-C10) does not approach zero until approximately 30 mol% sterol. From 1 to 25-30 mol% sterol, the enthalpy of the broad component of all of the sterol/SOPC mixtures increases in conjunction with decreasing sharp component enthalpy, thereafter decreasing toward zero at 50 mol% sterol. However, at 50 mol% sterol the broad component of the SOPC phase transition persists for the short side chain sterols, C-22, *i*-C5 and *n*-C3, and to a lesser degree in the longest side chain sterols *i*-C9 and *i*-C10, as well.

The variations in the transition temperatures of the sharp component of the SOPC chain-melting phase transition with sterol concentration are shown for both the *n*- and *iso*-series in Figures VI-5a and VI-5b, respectively. In both the *n*- and *iso*-series the transition temperature of the sharp component decreases progressively as a function of increasing sterol concentration. However, the extent of the decrease depends on sterol side chain length, with the shorter chain sterols inducing more dramatic decreases in the transition temperature. The transition temperature of the broad component of the SOPC phase transition for both the *n*- and *iso*-series exhibited similar sterol-dependent transition temperature shifts, as shown in Figures VI-6a and VI-6b, respectively. The incorporation of the shorter side chain sterols *n*-C3, *n*-C4, C-22, and *i*-C5 induced significant decreases in the transition temperature of the broad component, while increased incorporation of sterols *i*-C9 and *i*-C10 slightly increased the transition temperature of the broad component. Clearly, there is no significant difference between the temperature shifts induced by the *n*- and *iso*-series sterols for a given sterol side chain length and sterol concentration.

Lastly, the effect of sterol incorporation on the cooperativity of the overall chain-melting phase transition of SOPC is shown in Figures VI-7a (*n*-series) and VI-7b (*iso*-series). Increases in sterol incorporation dramatically increase the $\Delta T_{1/2}$ of the overall chain-melting phase transition of SOPC, and this increase in $\Delta T_{1/2}$ is independent of the *iso*- or *n*- structure. However, SOPC bilayers containing longer side chain sterols initially exhibit relatively small decreases in cooperativity compared to shorter-chain sterols. This situation reverses with progressive increases in sterol concentration, such that by 40 mol% sterol, the longer side chain sterols exhibit $\Delta T_{1/2}$ values as much as twice that observed in short-chain sterols.

DISCUSSION

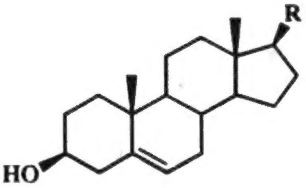
The overall chain-melting phase transition of SOPC clearly exhibits multiple melting components upon the addition of each sterol, indicative of the formation of sterol-poor (sharp transition) and sterol-rich (broad transition) phospholipid regions similar to that observed previously by DSC in cholesterol/PC and sterol/DPPC mixtures (Davis and Keough, 1983; McMullen *et al.*, 1993; Estep *et al.*, 1978; Mabrey *et al.*, 1978; McMullen and McElhaney, 1995). Alterations in the length of the sterol side chain has marked effects on the thermotropic phase behavior of each of these components. Both the sharp and broad components exhibited sterol-specific variations in enthalpy, transition temperature and cooperativity. However, while the DSC endotherms of sterol/SOPC bilayers respond to alterations in the length of the sterol side chain, we did not observe significant differences between terminally branched (*iso*) and unbranched (*n*) side chains of a given length. Our results correlate well with those of parallel FTIR (Chia *et al.*, 1993) and sterol oxidation studies (Slotte *et al.*, 1994), as well as prior DSC studies in cholesterol/PC and other sterol/DPPC mixtures (McMullen *et al.*, 1993, 1995). In conjunction with our earlier study, we also demonstrate that SOPC/sterol interactions depend primarily on the degree of hydrophobic mismatch. The formation of a crankshaft-kink in the SOPC *sn*-2 acyl-chains (Li *et al.*, 1994; Wang *et al.*, 1995), which only moderately shortens the average thickness of the SOPC bilayer, appears to permit more favorable sterol/SOPC contacts in the gel state than would be predicted for the much shorter, boomerang-like, *sn*-2 chain with a single C9-10 *cis*-kink (Huang, 1977; Wang *et al.*, 1995).

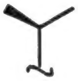
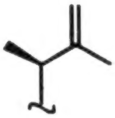
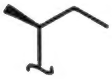
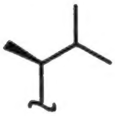
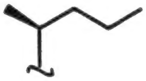
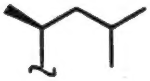
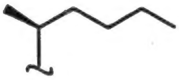
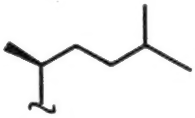
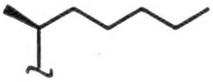
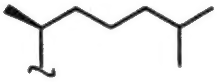
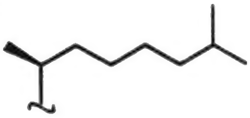
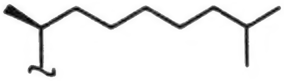
Both the sharp and broad components of the gel to liquid-crystalline phase transition of SOPC bilayers exhibit dramatic sterol-dependent shifts in transition temperature. The hydrophobic mismatch effect clearly accounts for the direction of these shifts in temperature. Briefly, hydrophobic mismatch represents the difference in mean hydrophobic length between the sterol and host phospholipid bilayer (McMullen *et al.*, 1993). The mean hydrophobic thickness of the host PC bilayer is defined as the mean hydrophobic length of the gel and liquid-crystalline phases while the hydrophobic thickness of the sterol molecule extends roughly from the C3 of the ring system to the tip of the alkyl side chain. Sterols with hydrophobic lengths greater than the mean hydrophobic length of the SOPC molecule will preferentially stabilize the gel state of SOPC bilayers, while sterols with short alkyl side chains will preferentially destabilize the gel state. McMullen *et al.* (1993, 1995) have shown previously that differences as small as 1.25 Å, or one methylene group, in hydrophobic length between the sterol and host lipid molecules produce significant shifts in the phase transition temperature of the broad component in DPPC bilayers. In DPPC bilayers, sterols containing alkyl side chains of seven carbon atoms or more produce increases in the temperature of the broad component of the chain-melting phase transition, while sterols containing 5 carbon atoms or less produce decreases in the broad component phase transition temperature (McMullen *et al.*, 1995). This result indicates that the mean hydrophobic length of the DPPC molecule in the bilayer is roughly equivalent to the hydrophobic length of a sterol having an alkyl side chain containing about six carbon atoms. In contrast, a significant decrease in the temperature of the broad component phase transition of SOPC bilayers is induced by all of the sterols studied here, except those with the longest alkyl chains (see Figure VI-8). This finding confirms that the mean hydrophobic thickness of SOPC bilayers must be greater than that of DPPC bilayers. In fact, the mean hydrophobic thickness of DPPC bilayers has been determined to be 32.9 Å (McMullen *et al.*, 1993; 1995) while that of SOPC bilayers has been estimated at about 36 Å, assuming that the oleoyl chain assumes predominately the crankshaft-kink conformation (Li *et al.*, 1994; Wang *et al.*, 1995). Thus our experimental results conform to the predictions of the hydrophobic mismatch model of sterol-phospholipid interactions, at least qualitatively. Moreover, the fact that SOPC bilayers in this study behave as if they are significantly thicker than DPPC bilayers lends some support to the postulate that the oleoyl chains of SOPC do adapt a more extended crankshaft-kink conformation in the gel-state, as postulated earlier (Li *et al.*, 1994; Wang *et al.*, 1995).

Interestingly, the effective stoichiometry of sterol/SOPC interactions also varies from that observed for sterol/DPPC systems, especially when the sterol side chain is significantly shorter than the isooctyl chain of cholesterol. We believe that the persistence of the sharp component of the chain-melting transition of SOPC to sterol levels beyond 20 mol%, and the persistence of the broad component to sterol levels beyond 50 mol%, indicates that these sterols do not mix ideally in the SOPC bilayer. The non-homogeneous dispersion of these sterols in the gel-state SOPC bilayer subsequently lowers the effective stoichiometry of sterol/SOPC interactions. Figure VI-9 clearly shows that the short side chain sterol C-22 is less effective in reducing the enthalpy of the main transition of the thicker SOPC bilayers than that of the thinner DPPC bilayers. The significant hydrophobic mismatch between short side chain sterols and the host SOPC bilayer creates an unfavorable free volume in the bilayer core which limits the miscibility of short side chain sterols in gel-state SOPC bilayers. Non-ideal mixing of SOPC and short side chain sterols also explains the relatively small increases in $\Delta T_{1/2}$ observed even with large increases in sterol concentration. The segregation of short side chain sterols into sterol-rich regions lowers the number of effective sterol/SOPC contacts, permitting a relatively more cooperative chain-melting phase transition than seen for SOPC bilayers with long side chain sterols.

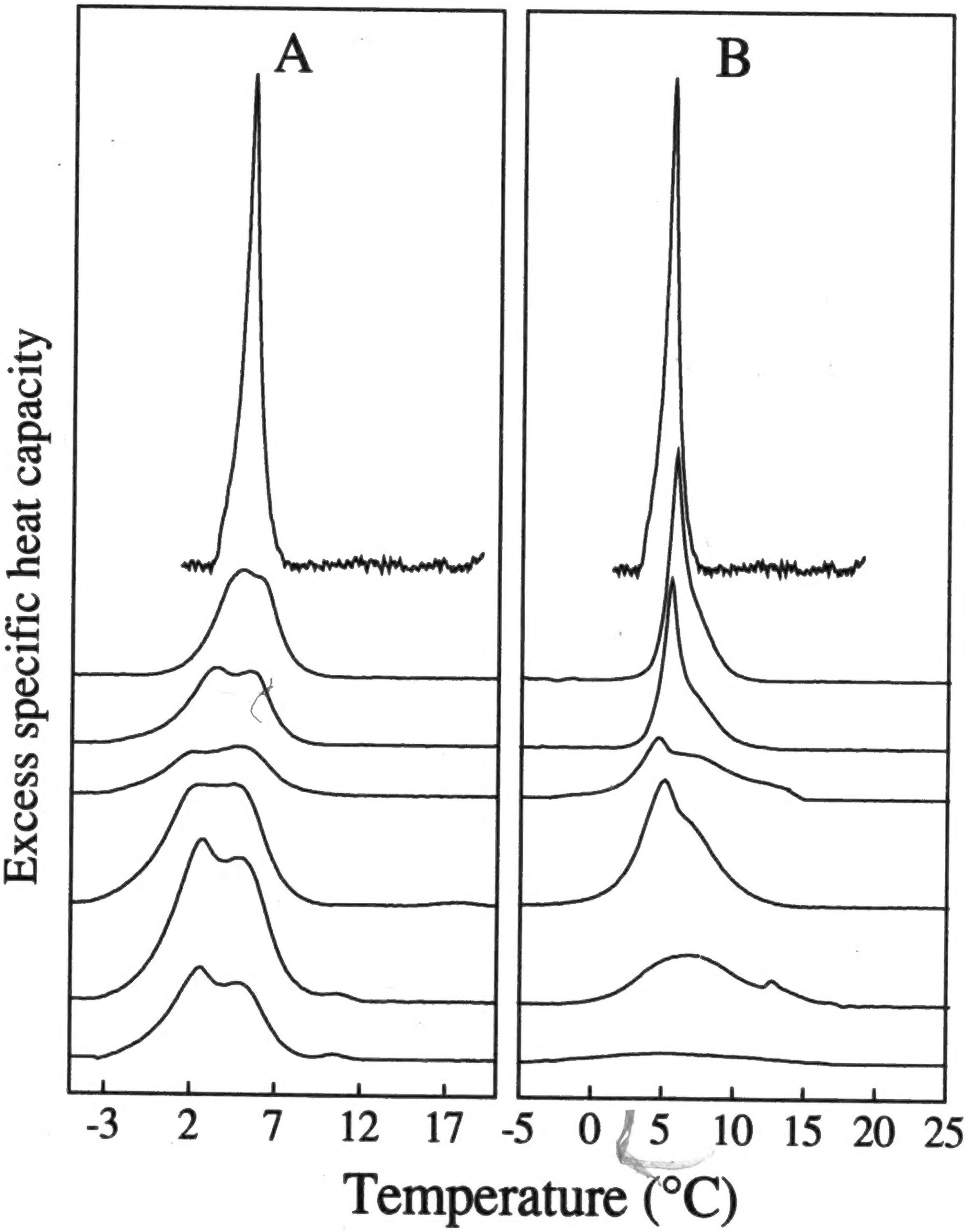
In summary, we have demonstrated that the thermotropic behavior and organization of sterol-containing SOPC mixtures depends primarily on the degree of hydrophobic mismatch between the sterol and the host PC bilayer. The degree of sterol-bilayer hydrophobic mismatch in turn clearly depends on the relative lengths (number of carbon atoms) of the alkyl chain of the sterol and the fatty acid chains of the phospholipid. Although this study provides no support for preferential interactions of cholesterol or its side chain analogues with the *cis*-monounsaturated PC hydrocarbon chains as proposed previously (Huang, 1977), the presence of unsaturation may be important in that it appears to determine the effective length of the PC bilayer, at least in the gel state. In particular, the crankshaft-kink conformation of the SOPC *sn*-2 acyl chain, which seems to be the most stable conformation within the bilayer, decreases the hydrophobic length of the SOPC molecule only slightly compared to its saturated analogue. Thus any change in hydrocarbon chain structure which alters the hydrophobic thickness of the bilayer will have dramatic effects on the thermotropic behavior and organization of sterol/PC mixtures.

Figure VI-1. Sterol side chain structures and their abbreviations as used in this paper.

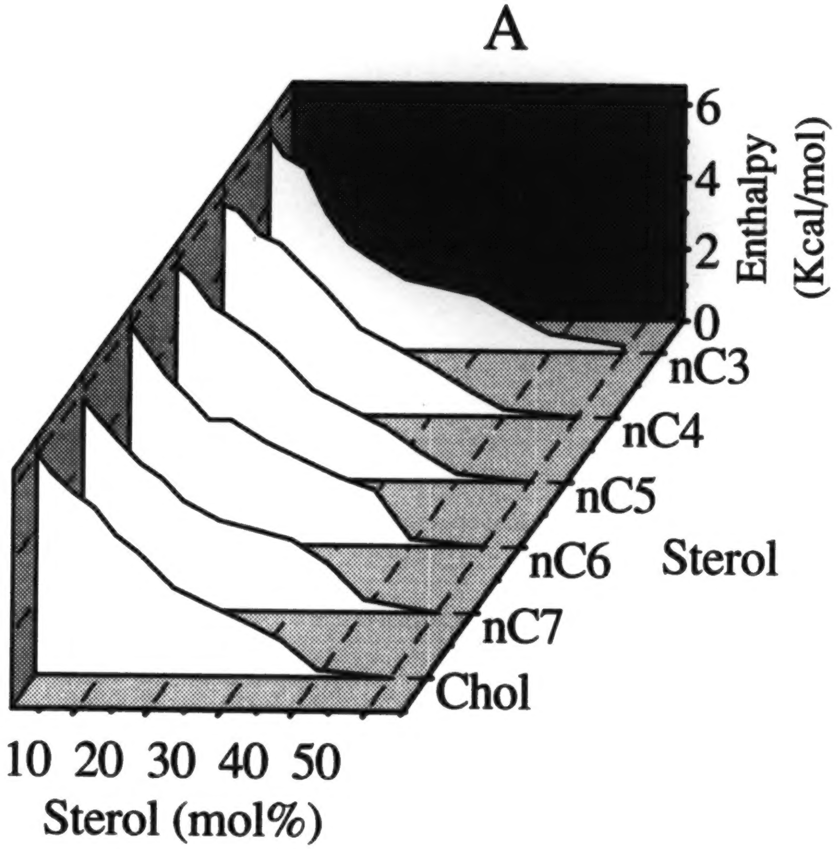


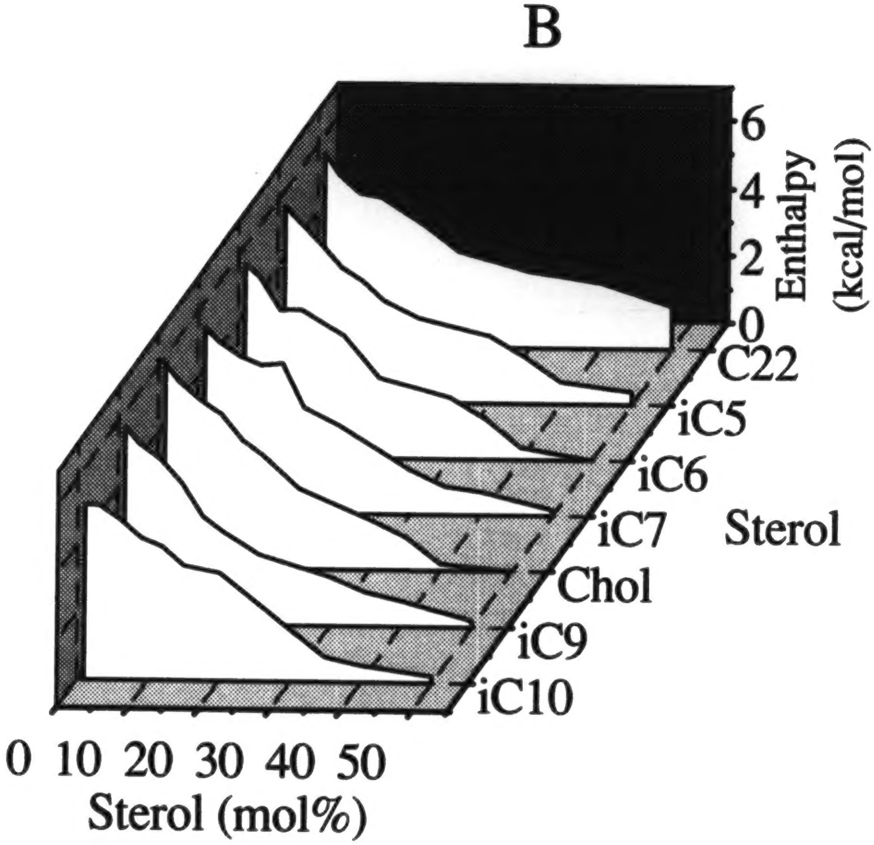
COMPOUND	CODE NAME	COMPOUND	CODE NAME
R =		R =	
	*n*-C3		C-22
	*n*-C4		*i*-C5
	*n*-C5		*i*-C6
	*n*-C6		*i*-C7
	*n*-C7		cholesterol
			*i*-C9
			*i*-C10

Figures VI-2a and VI-2b. Representative DSC thermograms of SOPC bilayers containing increasing amounts of (**A**) C-22 and (**B**) *i*-C10. Sterol concentrations, shown in descending order on the figure, are 0, 3, 6, 9, 12, 20, and 40 mol%. Sterol concentrations done but not shown are 15, 25, and 30 mol%.

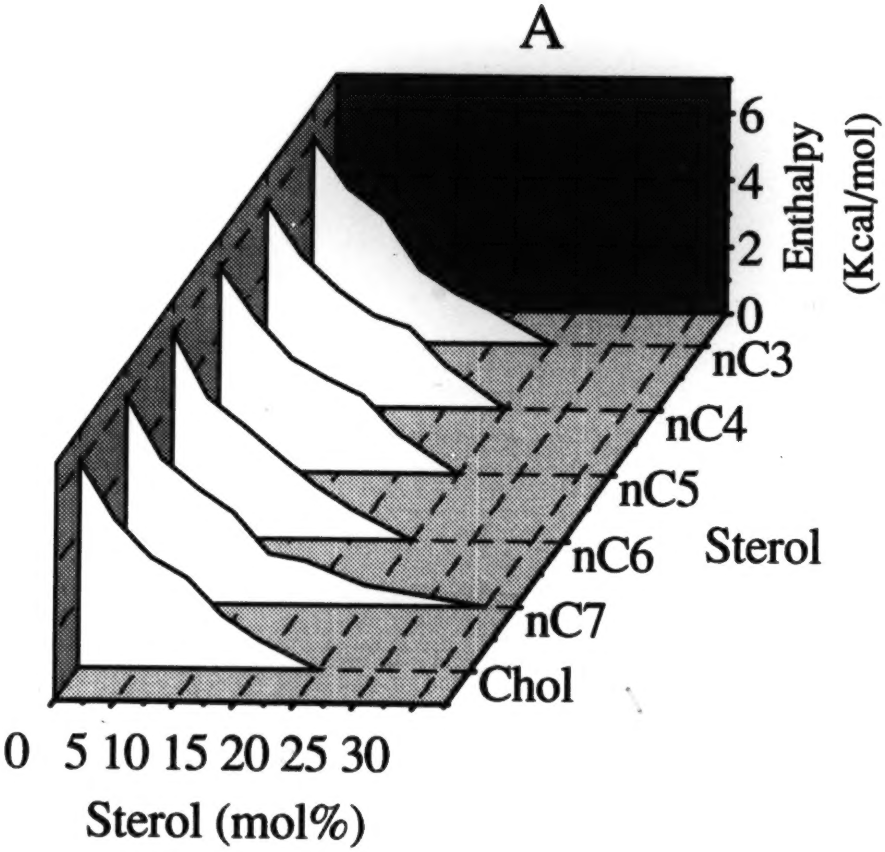

A
B
Excess specific heat capacity
-3
2
7
12
17
-5
0
5
10
15
20
25
Temperature (°C)

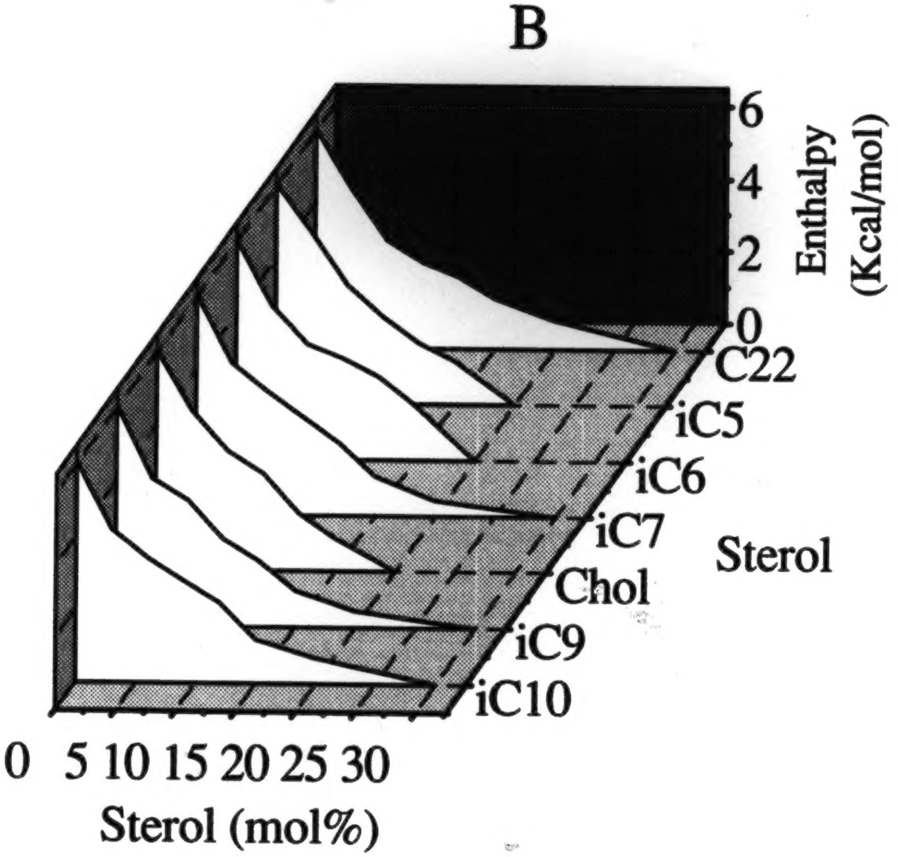
Figures VI-3a and VI-3b. Three dimensional plot of the effect of increasing sterol concentration on the overall enthalpy of the SOPC main transition for the (**A**) *n*- and (**B**) *iso*-series of sterols. Sterols and their respective concentrations and are shown on the figure.


A
6
4
2
0
Enthalpy
(Kcal/mol)
nC3
nC4
nC5
nC6
nC7
Chol
Sterol
10 20 30 40 50
Sterol (mol%)

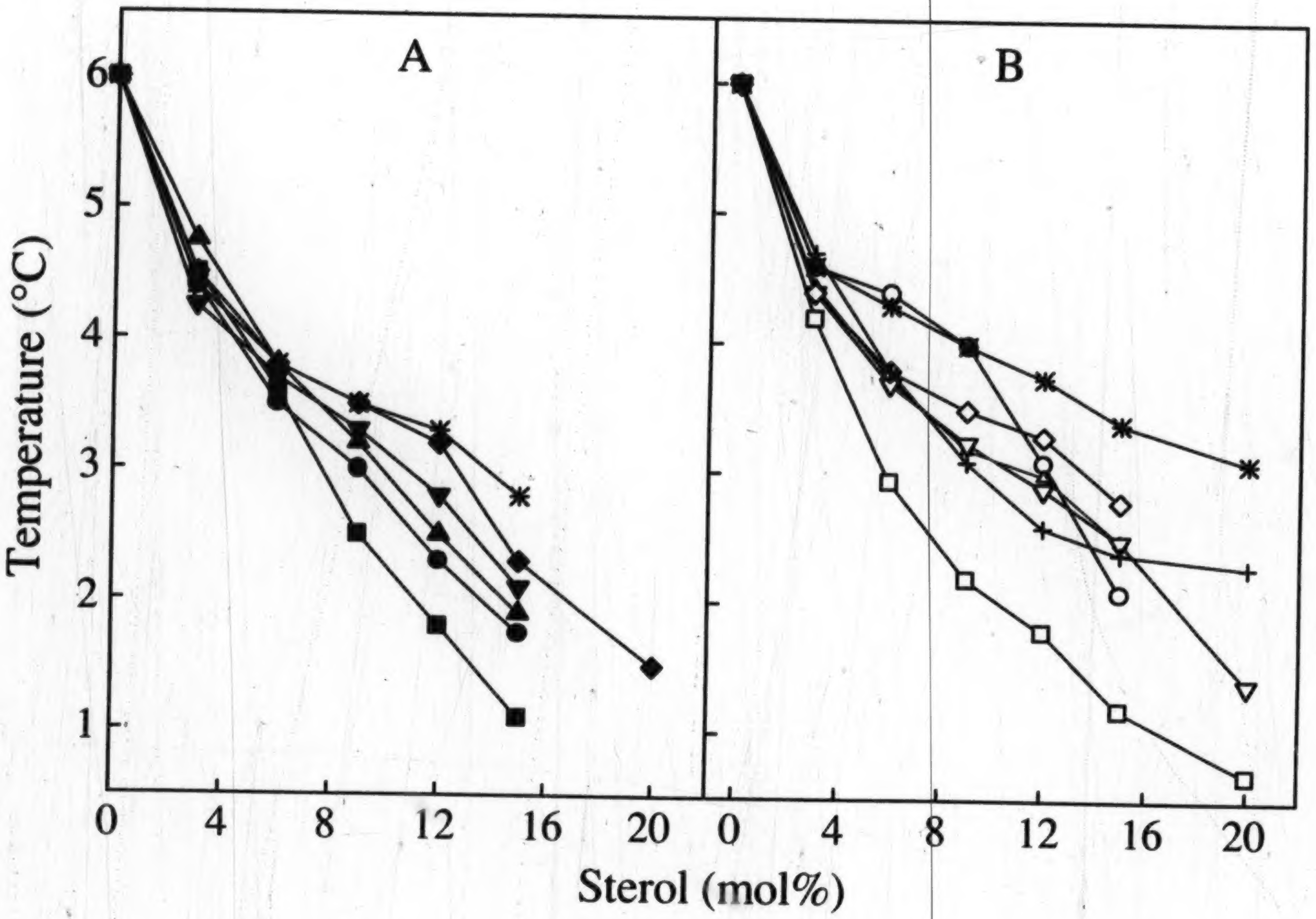

B
6
4
2
0
Enthalpy
(kcal/mol)
C22
iC5
iC6
iC7
Chol
iC9
iC10
Sterol
0
10
20
30
40
50
Sterol (mol%)

Figures VI-4a and VI-4b. Sharp component enthalpy of sterol/SOPC mixtures as a function of increasing sterol concentration for the **(A)** *n*- and **(B)** *iso*-series sterols. Sterols and their respective concentrations are shown on the figure.

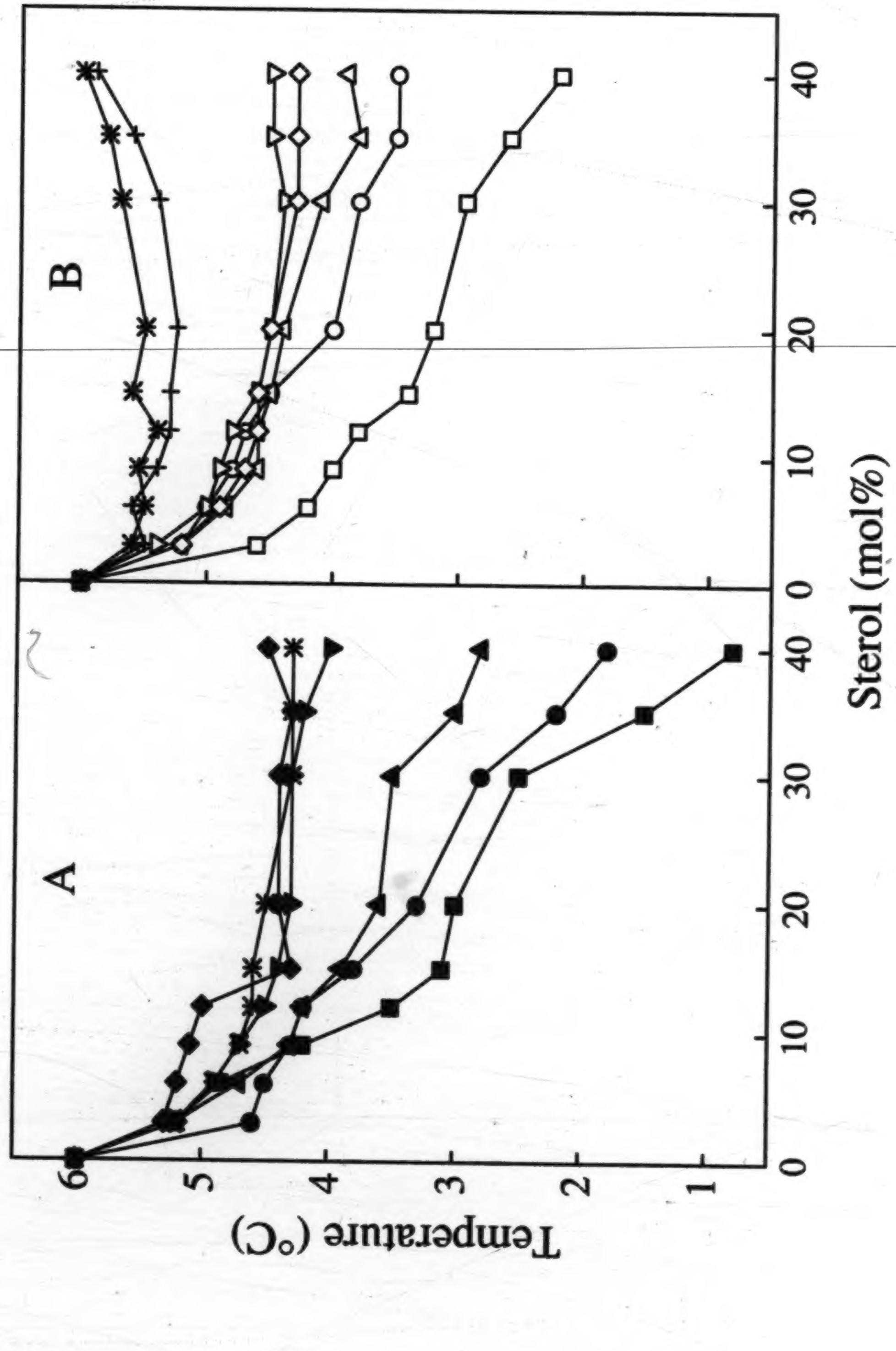

A
6
4
2
0
Enthalpy
(Kcal/mol)
nC3
nC4
nC5
nC6
nC7
Chol
Sterol
0
5
10
15
20
25
30
Sterol (mol%)


B
6
4
2
0
Enthalpy (Kcal/mol)
C22
iC5
iC6
iC7
Chol
iC9
iC10
Sterol
0
5
10
15
20
25
30
Sterol (mol%)

Figures VI-5a and VI-5b. Sharp component transition temperature of sterol/SOPC mixtures as a function of increasing sterol concentration: (**A**) *n*-C3 (■); *n*-C4 (●); *n*-C5 (▲); *n*-C6 (▼); *n*-C7 (◆); Chol (*), (**B**) C-22 (□); *i*-C5 (○); *i*-C6 (Δ); *i*-C7(∇); Chol (◊); *i*-C9 (+); *i*-C10 (*). All values +/- 0.40 °C.


A
B
Temperature (°C)
1
2
3
4
5
6
Sterol (mol%)
0
4
8
12
16
20
0
4
8
12
16
20

Figures VI-6a and VI-6b. Broad component transition temperature of sterol/SOPC mixtures as a function of increasing sterol concentration: **(A)** *n*-C3 (■); *n*-C4 (●); *n*-C5 (▲); *n*-C6 (▼); *n*-C7 (◆); Chol (*), **(B)** C-22 (□); *i*-C5 (○); *i*-C6 (Δ); *i*-C7(∇); Chol (◊); *i*-C9 (+); *i*-C10 (*).Up to 20 mol%, values +/- 0.40 °C; thereafter +/- 1.0 °C.


A
B
Temperature (°C)
Sterol (mol%)
6
5
4
3
2
1
0
10
20
30
40

Figures VI-7a and VI-7b. Overall $\Delta T_{1/2}$ of sterol/SOPC endotherms as a function of increasing sterol concentration: (**A**) *n*-C3 (■); *n*-C4 (●); *n*-C5 (▲); *n*-C6 (▼); *n*-C7 (◆); Chol (*), (**B**) C-22 (❑); *i*-C5 (○); *i*-C6 (Δ); *i*-C7(∇); Chol (◊); *i*-C9 (+); *i*-C10 (*). Up to 30 mol%, $\Delta T_{1/2}$ values +/- 0.40 °C; for 40 mol% +/- 1.0 °C.

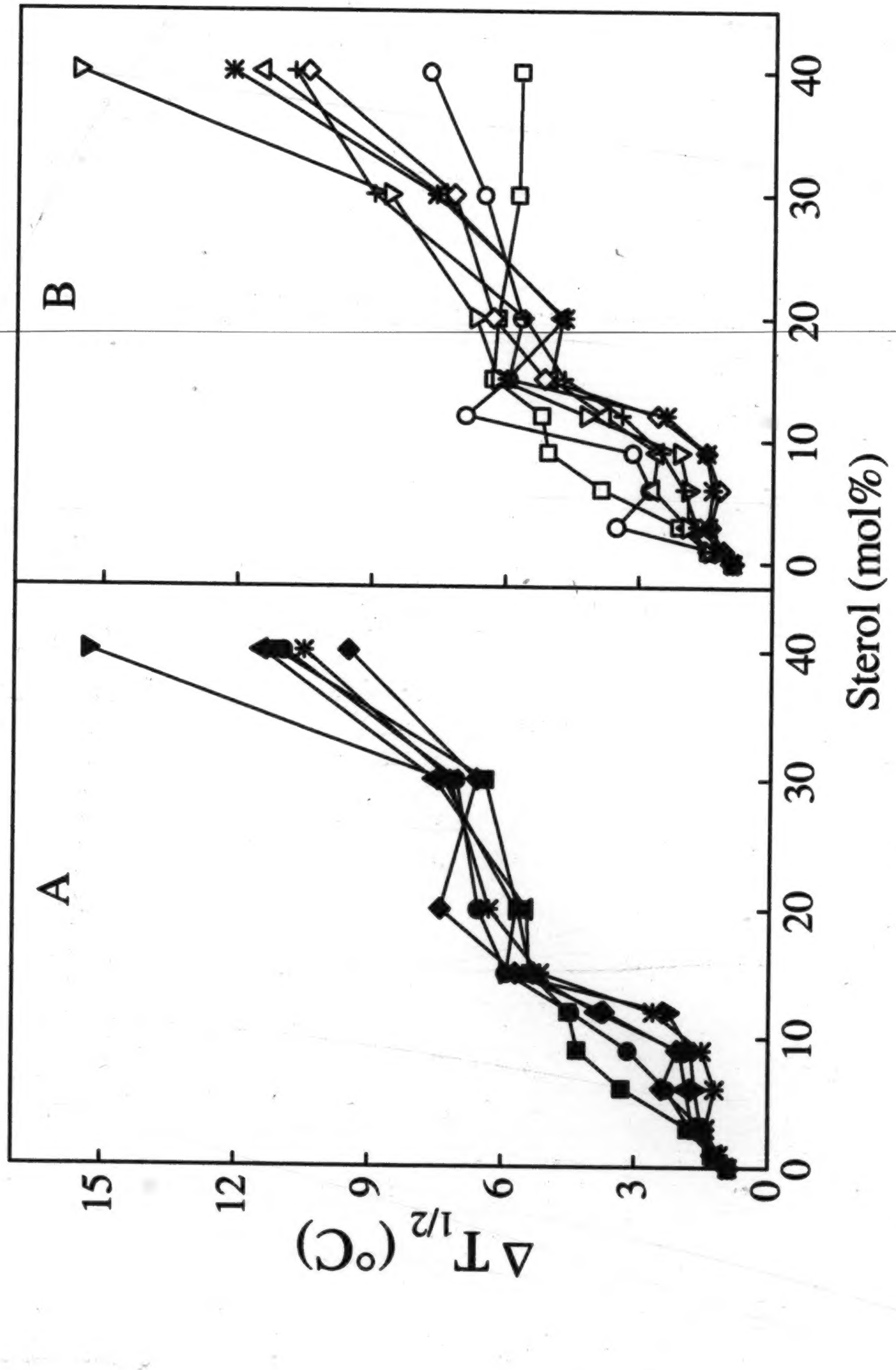

A
B
$\Delta T_{1/2}$ (°C)
0
3
6
9
12
15
0
10
20
30
40
0
10
20
30
40
Sterol (mol%)

Figure VI-8. Broad component transition temperature shifts for both SOPC and DPPC bilayers containing cholesterol, C-22 and *i*-C10 sterols as a function of increasing sterol concentration: cholesterol/SOPC (▲); C-22/SOPC (■); *i*-C10/SOPC (●); cholesterol/DPPC (▽); C-22/DPPC (□); *i*-C10/DPPC (○). All values +/- 0.40 °C.

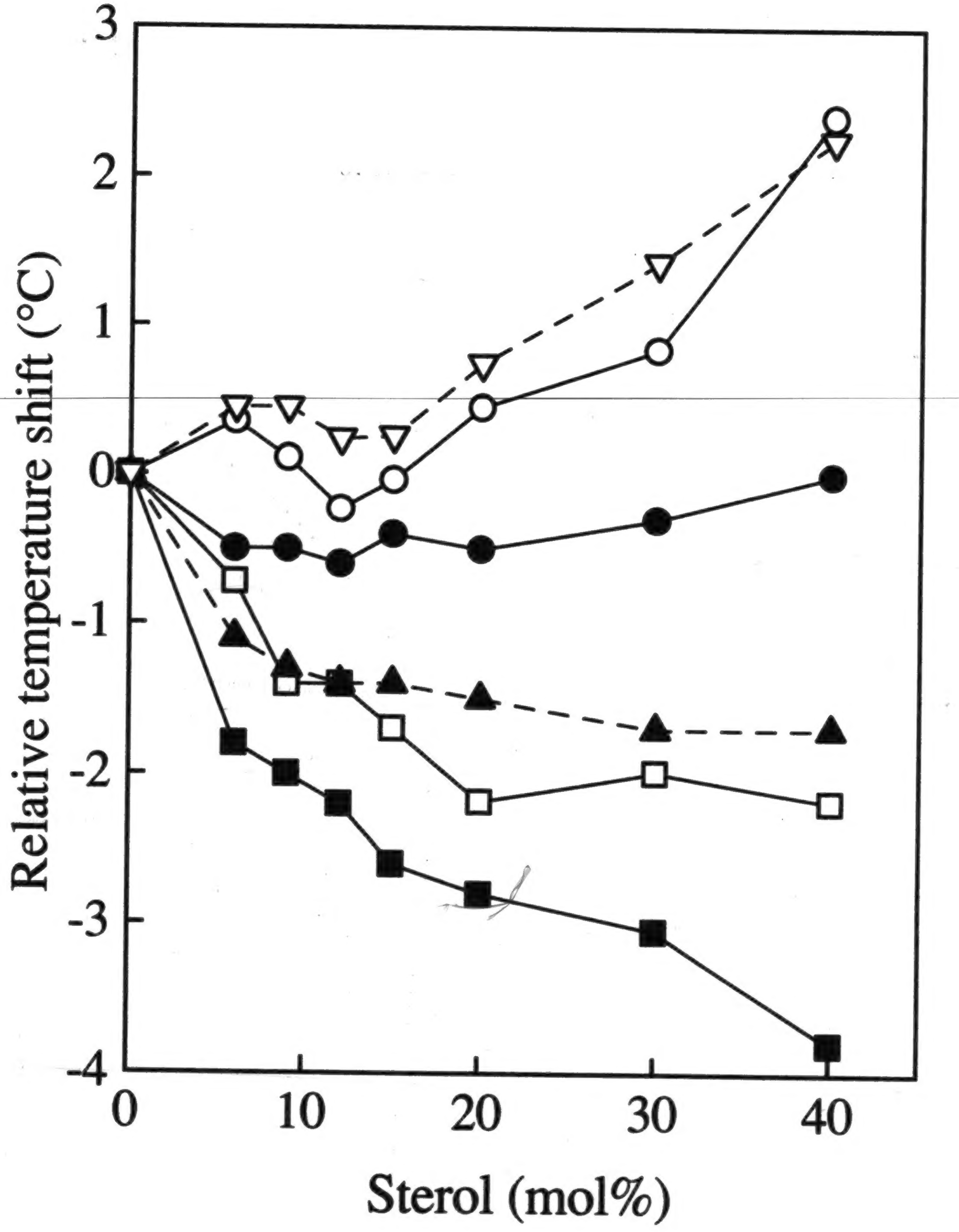
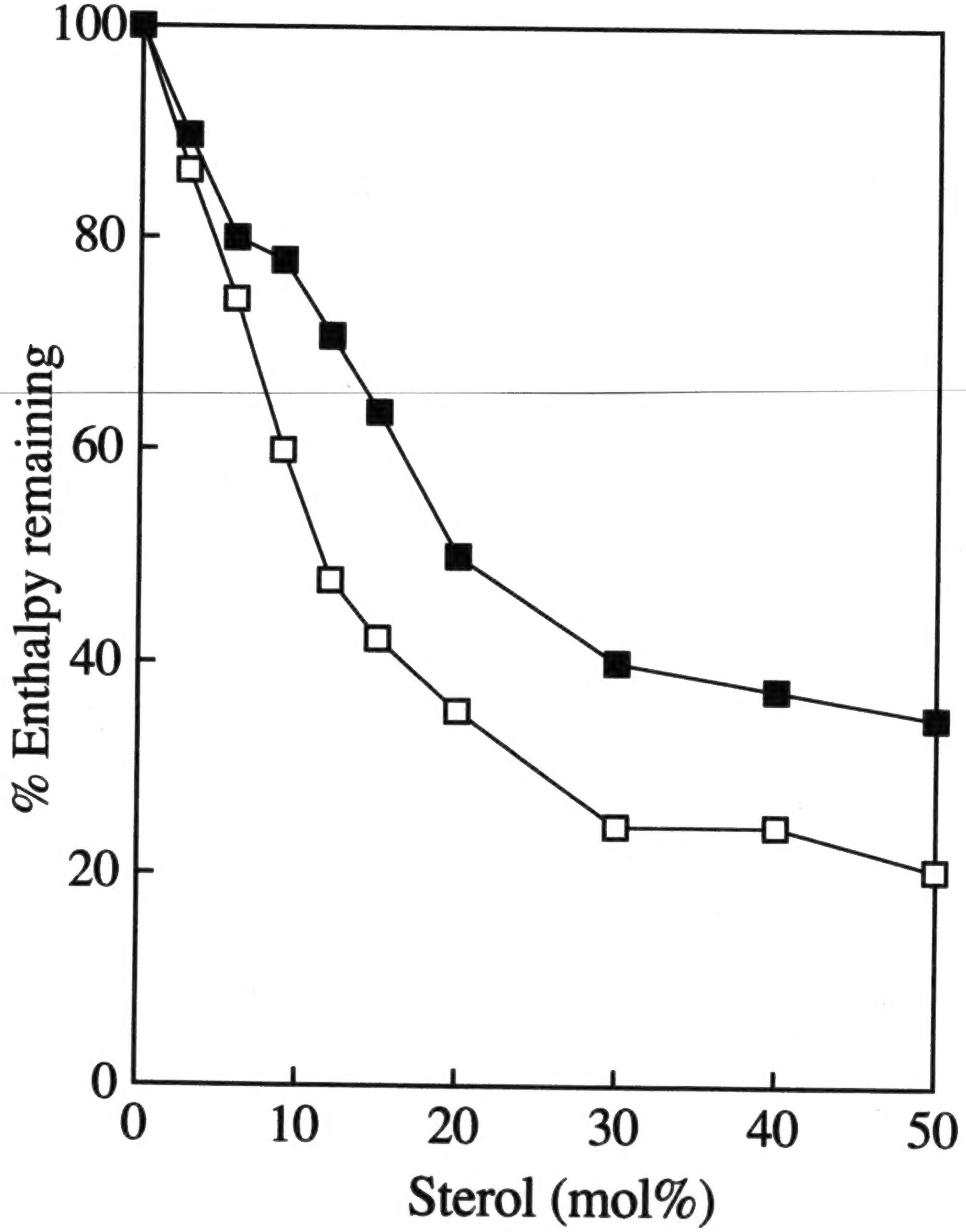

Relative temperature shift (°C)
3
2
1
0
-1
-2
-3
-4
0
10
20
30
40
Sterol (mol%)

Figure VI-9. Plots of the % remaining enthalpy of the overall chain-melting transition for SOPC and DPPC bilayers as a function of increasing C-22 sterol concentration: C-22/SOPC (■); C-22/DPPC (❑). All values +/- 5.0%.


100
80
60
40
20
0
% Enthalpy remaining
0
10
20
30
40
50
Sterol (mol%)

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CHAPTER VII. CALORIMETRIC AND SPECTROSCOPIC STUDIES OF THE EFFECTS OF CHOLESTEROL ON THE THERMOTROPIC PHASE BEHAVIOR AND ORGANIZATION OF A HOMOLOGOUS SERIES OF LINEAR SATURATED PHOSPHATIDYLETHANOLAMINE BILAYERS[7]

INTRODUCTION

Cholesterol, or a closely related sterol, is an essential constituent and an important structural component of the plasma membrane of virtually all eukaryotic cells (Nes and McKean, 1977). Cholesterol is known to be a potent modulator of the bulk physical properties of biological membranes (Demel and de Kruijff, 1976; Razin and Rottem 1978; Yeagle 1988; McMullen and McElhaney, 1996) and the presence of cholesterol or cholesterol-rich phospholipid domains in membranes has been postulated to directly affect protein conformation and function. (Cheng *et al.*, 1986; Yeagle, 1988, 1991; Narayanaswami and McNamee, 1993; Fernandez-Ballester *et al.*, 1994; Lasalde *et al.*, 1995; Cornelius, 1995; Schroeder *et al.*, 1995). In addition, the relative affinity of cholesterol for different phospholipids in the host membrane may govern the lateral distribution of cholesterol within the membrane, and cholesterol-rich phospholipid domains may be important to the sorting and localization of membrane proteins within eukaryotic cells (Thompson *et al.*, 1992; van Meer, 1993; Bretscher and Munro, 1993; Welti and Glaser, 1994; Schroeder *et al.*, 1995). However, while it is clear that cholesterol is vital to the structure and function of biological membranes, our understanding of the molecular basis of cholesterol-phospholipid interactions remains incomplete (McMullen and McElhaney, 1996).

Since the pioneering study by Ladbrooke *et al.* (1968), the phase behavior and organization of cholesterol-containing model and biological membranes have been the subject of extensive study (Yeagle, 1988; McMullen and McElhaney, 1996). However, cholesterol/DMPC and cholesterol/DPPC mixtures account for the majority of either HS-

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[7] A version of this chapter has been submitted for publication to *Biophys J.* McMullen, T.P.W., Lewis, R.N.A.H. and McElhaney, R.N. (1996).

or LS-DSC studies on the thermotropic phase behavior and organization of cholesterol/phospholipid mixtures. (Yeagle, 1988; McMullen *et al.*, 1993). Conversely, there are relatively few studies of the thermotropic phase behavior of cholesterol/PE mixtures, despite the fact that PE's are a major component of eukaryotic plasma membranes (Ansell and Spanner, 1982; Koynova and Caffery, 1994). Of the few studies that have examined the effect of cholesterol on the thermotropic phase behavior and organization of PE bilayers, all but two of which rely exclusively on DSC, the results vary markedly. For example, the addition of cholesterol was reported either to markedly reduce (POPE and DEPE), moderately reduce (DPPE, EYPE) or have little effect on (DMPE) the chain-melting phase transition temperature of the host PE bilayer (van Dijck *et al.*, 1976; Blume, 1980; Epand and Bottega, 1987; Cheetham *et al.*, 1989). In addition, the levels of cholesterol required to abolish the cooperative chain-melting phase transition of these same PE bilayers varies from as little as 30 mol% cholesterol to levels exceeding 50 mol% cholesterol. There is also evidence for solid phase cholesterol in cholesterol-containing PE bilayers at cholesterol levels as low as 20 to 30 mol%, particularly in those bilayers where one or both of the PE hydrocarbon chains is unsaturated (van Dijck *et al.*, 1976; Blume, 1980; Epand and Bottega, 1987; Cheetham *et al.*, 1989). However, there is no apparent relationship between the solubility of cholesterol and the resulting effect of cholesterol on the thermotropic phase behavior of the host bilayer, as cholesterol still appears to abolish the gel to liquid-crystalline phase transition of those PE bilayers with which it is supposedly the least soluble (Cheetham *et al.*, 1989). Furthermore, in studies of monotectic DOPE/DPPC and DOPC/DMPE mixtures, cholesterol was reported to abolish the chain-melting phase transition of the PC component at cholesterol levels considerably lower than for the PE component, thus indicating that cholesterol preferentially associates with PC's (van Dijck *et al.*, 1976; Demel *et al.*, 1977; Cullis *et al.*, 1978; Tilcock *et al.*, 1982). However, cholesterol does not appear to have a preference for either PC or PE in non-monotectic DMPE/DMPC mixtures (Blume, 1980). Finally, the observed thermotropic phase behavior and organization of cholesterol/PE mixtures also appears to vary when studied by either calorimetry or NMR spectroscopy. When Blume and Griffin (1982) attempted, for the first and only time thus far, to utilize both DSC and ^{2}H-NMR to develop a temperature/composition diagram for cholesterol/DPPE mixtures, they found that the phase boundaries varied by as much as 25 °C between the two techniques. Moreover, ^{2}H-NMR revealed metastable behavior not observed by DSC, especially at high cholesterol concentrations. These differences were ascribed to the inability of DSC to monitor the poorly cooperative phase transitions of the small cholesterol-rich DPPE domains which were postulated to exist in this system.

Recently, we demonstrated that the phase behavior and organization of both saturated and unsaturated PC bilayers containing cholesterol or cholesterol analogues varies considerably with changes in PC hydrocarbon chain length as well as with the length of the sterol C17 alkyl side chain (McMullen *et al.*, 1993, 1994, 1995; Vilchèze *et al.*, 1996). In particular, the miscibility of the sterol, and thus the magnitude of the effect of the sterol on the thermotropic phase behavior of the host PC bilayer, was found to decrease markedly with increasing hydrophobic mismatch between the sterol and the phospholipid bilayer. Similarly, the interactions of SPM and GalCer with cholesterol, and subsequent changes in the molecular area of cholesterol/SPM or cholesterol/GalCer mixtures, depend on the hydrocarbon chain composition and phase state of the host phosphosphingolipid or glycosphingolipid bilayer (Ali *et al.*, 1994; Smaby *et al.*, 1995). Thus, to properly address the issue of the effect of phospholipid headgroup structure on the phase behavior of cholesterol-PE mixtures, we have systematically investigated the thermotropic phase behavior and organization of cholesterol/PE bilayers with hydrocarbon chain lengths varying from 14 to 18 carbons using HS-DSC, FTIR and ^{31}P-NMR spectroscopy. This study is designed to complement our prior study of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated PC's (McMullen *et al.*, 1993). Briefly, we find that the miscibility of cholesterol, or the effective stoichiometry of cholesterol-PE interactions, decreases with decreasing temperature and increasing PE hydrocarbon chain length. This is in marked contrast to the temperature- and chain length-independent stoichiometry of cholesterol-PC interactions revealed in our prior study (McMullen *et al.*, 1993). We also find coexisting cholesterol-poor crystalline and cholesterol-rich gel and liquid-crystalline phospholipid domains in cholesterol/PE mixtures at cholesterol concentrations of up to 50 mol%. The relative proportion of these cholesterol-rich and cholesterol-poor phospholipid domains depends on the temperature and level of cholesterol. As a result, the observed effect of cholesterol on the phase behavior and organization of the host PE bilayer mixtures will also vary with the technique employed because of differences in the thermal history of the sample. This fact may, at least in part, account for the differences in the effect of cholesterol on the phase behavior and organization of PE bilayers noted in prior studies. Overall, this study has important implications for subsequent studies of cholesterol-phospholipid interactions in binary and higher order cholesterol/phospholipid mixtures of varying phospholipid headgroup and hydrocarbon chain composition.

MATERIALS AND METHODS

The PEs used in these experiments were purchased from Avanti Polar Lipids (Alabaster, AB) and checked for purity by TLC using chloroform/methanol/ammonia (50:50:4, by vol.) as the developing solvent followed by spraying with 2% K_2CrO_4 in 60% sulfuric acid and charring. Each PE gave a single spot on the developed TLC plates. The cholesterol was also purchased from Avanti Polar Lipids and recrystallized from ethanol before use. Initially, a known amount of DMPE or cholesterol were dissolved in chloroform/methanol (2:1, vol/vol) to make stock solutions from which the cholesterol/DMPE dispersions would be quantitatively mixed. To ensure that the stock solutions gave homogenous cholesterol/DMPE mixtures, we evaluated the chemical composition and thermotropic behavior of cholesterol/DMPE mixtures prepared from stock solutions of chloroform, chloroform/methanol (2:1, vol/vol), chloroform/methanol (1:2, vol/vol) and methanol as well as lyophilized from benzene. We found that the best procedure for producing homogenous mixtures of DMPE and cholesterol required chloroform/methanol (2:1, vol/vol) stock solutions or lyophilization from benzene which, after mixing, were heated to approximately 40 to 50 °C under N_2 to remove the solvent, then dried under vacuum overnight. In the case of cholesterol/DPPE and cholesterol/DSPE mixtures, the amount of the PE was gravimetrically determined and a stock solution of cholesterol was added. The heated chloroform/methanol mixtures were dried under N_2, then dissolved in benzene with heating to ensure a clear and fully codissolved PE/cholesterol mixture, and then lyophilized. When carefully followed this protocol provides fully reproducible thermograms. Failure to lyophilize from benzene or to heat the chloroform/methanol PE/cholesterol mixture would often require repeated heating and cooling DSC runs upon hydration to achieve consistent thermogram profiles. We also attempted to study cholesterol/DAPE mixtures, however, despite trying both lyophilization or chloroform/methanol dissolution protocols, we were unable to prepare homogenous mixtures as defined by reproducible heating and cooling DSC scans (see discussion).

For all of the HS-DSC experiments the dried cholesterol/PE mixtures were dispersed in deionized water, heated to approximately 10-20 °C above the phase transition of the mixture, and then vortexed to give a multilamellar suspension. The thermotropic phase behavior of PE and cholesterol/PE mixtures suspended with (50 to 100 mM Tris or phosphate buffers, with 10 mM EDTA and/or 100 mM NaCl) or without buffered deionized water was virtually identical. The HS-DSC thermograms for the cholesterol/PE suspensions were recorded with a Hart high-sensitivity differential scanning calorimeter (Provo, UT). All of the samples, both with and without cholesterol, were all kept overnight at 2 °C, then scanned by HS-DSC at 10 °C/hr (unless otherwise noted in the results section). The amount of PE used for HS-DSC was progressively increased from 0.5 mg for pure PE bilayers to 20 mg for PE samples containing 45 or 50 mol% cholesterol. We have shown previously that this protocol is required to accurately monitor the broad, low-enthalpy phase transitions observed at higher cholesterol concentrations (McMullen *et al.*, 1993). The Hart calorimeter was calibrated using solid standards from Hart Scientific as well as aqueous lipid samples synthetically prepared and purified within this laboratory using methods previously shown to provide highly pure samples (Lewis *et al.*, 1985). Sample runs were repeated at least three times to ensure reproducibility. For all mixtures phospholipid and cholesterol degradation was monitored by TLC and no degradation products were observed. Moreover, sequential HS-DSC runs were completely reproducible, supporting the absence of chemical degradation in our samples after HS-DSC or spectroscopic analysis. The analysis and the decomposition of the HS-DSC endotherms was done using Microcal's Origin (Northhampton, MA) and DA-2 software. Briefly, the procedure for HS-DSC decomposition is based on the assumption that the observed thermograms can be approximated as a linear combination of multiple, independent two-state transitions (Estep *et al.*, 1978; Mabrey *et al.*, 1978; McMullen *et al.*, 1993). The curve broadening is expressed in terms of the van't Hoff enthalpy, which is evaluated by the equation $\Delta H_{vH} = 4RT_m^2(c_{max}/\Delta q)$, where c_{max} is the excess specific heat capacity and Δq is the area under the curve.

For FTIR spectroscopic analysis, the cholesterol/PE dispersions were prepared identical to that described above for the HS-DSC experiments, with the exception that the samples were suspended in D_2O. The samples were then placed between CaF_2 windows containing a 25-um spacer and mounted in a cell holder attached to a computer-controlled circulating water bath. FTIR spectra were recorded with a Digilab (Cambridge, MA) FTS-40 Fourier transform infrared spectrometer. Temperature-induced shifts in the absorption maxima and bandwidth of the CH_2 symmetric and asymmetric stretching bands near 2850 cm^{-1} and 2920 cm^{-1}, respectively, were used to monitor changes in the conformational state of the hydrocarbon chains of the PE molecules (Lewis and McElhaney, 1993; Mendelsohn and Mantsch, 1986; Snyder, 1961, 1967; Mantsch and McElhaney, 1991), while alterations in the frequency of the CH_2 scissoring band near 1468 cm^{-1} indicated changes in the solid-state PE hydrocarbon chain packing (Lewis and McElhaney, 1993; Snyder, 1961; 1979). In addition, we monitored the absorption of the phospholipid carbonyl stretching band centered at 1735-1745 cm^{-1}, which assisted in the identification of PE bilayers exhibiting dehydrated interfaces typical of crystalline hydrocarbon chain packing (Lewis and McElhaney, 1993). The data was processed using computer programs developed by the National Research Council of Canada.

Pure PE or cholesterol/PE mixtures for ^{31}P-NMR were prepared as outlined for the HS-DSC experiments, except that the amount of PE in the sample was typically 30 to 50 mg. Proton-decoupled ^{31}P-NMR spectra were acquired with a Varian (Palo Alto, CA) Unity-300 spectrometer operating at 121.42 MHz for ^{31}P. The data acquisition and data processing protocols were the same as the single-pulse, direct-excitation techniques described by Lewis *et al.* (1988). After completion of the NMR experiments, the samples were checked for degradation by both TLC and HS-DSC. No significant degradation or alterations in sample thermotropic behavior were observed.

RESULTS

Thermotropic phase behavior of pure phosphatidylethanolamines.

Recently Lewis and McElhaney (1993), using HS-DSC and FTIR and ^{31}P-NMR spectroscopy, systematically examined the phase behavior of a homologous series of PE bilayers with hydrocarbon chain lengths varying from 12:0 to 20:0 carbons. In the absence of cholesterol, the polymorphic phase behavior of fully hydrated PEs may exhibit one or two of three different transitions, depending on the length of the hydrocarbon chain and whether or not the sample was previously annealed at low temperatures (Lewis and McElhaney, 1993 and references therein). All fully hydrated unannealed PEs undergo a L_β to L_α transition at a characteristic temperature (see Figures VII-1, 2 and 3). However, the shorter chain PE's (10:0 to 16:0), having undergone sufficient low temperature incubation to anneal the sample (see materials and methods), will form an L_c phase, characterized by dehydrated headgroups with extensive inter-headgroup hydrogen bonding and tight hydrocarbon chain packing, which will convert directly to the L_α phase in a single, highly energetic L_c/L_α phase transition. For the longer chain PEs (>DPPE), the L_c phases require longer and more complicated incubation regimes and in fully hydrated DSPE bilayers, the L_c phase was not observed within several months. Note that for DMPE and DPPE, the L_c phase, once formed, is more thermally stable than the L_β phase and undergoes hydrocarbon chain-melting at a higher temperature. With increasing chain length the enthalpy for both transitions increases. This information on the thermotropic phase behavior of pure PEs will be essential to understanding the effects of cholesterol on PE bilayers described below.

Effect of cholesterol on the thermotropic phase behavior and organization of DMPE, DPPE and DSPE bilayers: Calorimetric studies.

The effect of cholesterol on the thermotropic phase behavior of PE bilayers clearly depends on the hydrocarbon chain length of the PE molecule. Shown in Figure VII-1 are the heating (left panel) and cooling (right panel) HS-DSC scans of DMPE bilayers containing progressively increasing levels of cholesterol. In the absence of cholesterol and in both the heating and cooling modes, DMPE bilayers undergo a highly cooperative L_β to L_α phase transition at approximately 50 °C with an enthalpy value of approximately 6.2 kcal/mol. Upon the addition of cholesterol at concentrations up to 30 mol%, the temperature (Figure VII-4), cooperativity (Figure VII-1), and the enthalpy (Figure VII-5) of the DMPE chain-melting phase transition decrease notably on both heating and cooling. From 5 to 15 mol% cholesterol, the symmetrical heating and cooling endotherms of cholesterol/DMPE mixtures are both well fit by two overlapping components (results not shown), consistent with the existence of cholesterol-rich and cholesterol-poor phospholipid domains (Estep *et al.*, 1978; Blume, 1980; Vist and Davis, 1990; McMullen *et al.*, 1993; McMullen and McElhaney, 1995). Thus, below 30 mol% cholesterol, the thermotropic behavior of the cholesterol/DMPE mixtures is virtually identical in the heating and cooling modes. However, as the level of cholesterol increases to 40 and 50 mol% cholesterol, the thermotropic phase behavior of the cholesterol/DMPE mixtures depends on whether monitored in the heating or cooling mode. In the heating mode we observe a marked increase in the overall enthalpy (Figure VII-5) and cooperativity (Figure VII-1) of the endotherms of DMPE bilayers containing 35 and 50 mol% cholesterol. The temperature (Figure VII-4) and enthalpy (Figure VII-5) values obtained from the multicomponent endotherms of DMPE bilayers with 35 and 50 mol% cholesterol on heating are consistent with a combination of overlapping L_β/L_α and L_c/L_α phase transitions, both well documented in pure DMPE bilayers by Lewis and McElhaney (1993). Since the L_c phase of DMPE bilayers contains arrays of pure phospholipids (Lewis and McElhaney, 1993), cholesterol must be excluded from the crystalline PE domains. Conversely, in the cooling mode, the temperature (Figure VII-4), enthalpy (Figure VII-5) and cooperativity (Figure VII-1) of the L_α/L_β phase transition progressively decrease and the transition is completely abolished by 50 mol% cholesterol. No other endotherms were noted on cooling. Overall, it is clear that the effect of cholesterol on the thermotropic phase behavior of DMPE bilayers is significantly different from that observed in corresponding cholesterol/DMPC mixtures, especially at high cholesterol concentrations (McMullen *et al.*, 1993).

Shown in Figure VII-2 are the representative HS-DSC heating (left panel) and cooling (right panel) scans for DPPE bilayers containing progressively increasing amounts of cholesterol. In the absence of cholesterol and in both the heating and cooling modes, DPPE bilayers exhibit a L_β to L_α phase transition at approximately 64.5 °C with an enthalpy value of 8.7 kcal/mol. Upon the incorporation of cholesterol, the chain-melting transition temperature (Figure VII-4), cooperativity (Figure VII-2) and enthalpy (Figure VII-5) of DPPE bilayers all decrease markedly. However, unlike the corresponding cholesterol/DMPE mixtures containing up to 15 mol% cholesterol, the endotherms of cholesterol/DPPE mixtures exhibit significant broadening on the low-temperature side and can not be well fit by combinations of single cholesterol-rich and -poor components. Moreover, while the chain-melting transition endotherms obtained upon heating and cooling are similar up to 20 mol% cholesterol, they do not correspond above 20 mol% cholesterol and the differences intensify with increasing cholesterol levels. In the heating mode, DPPE bilayers with cholesterol levels exceeding 20 mol% clearly exhibit a multicomponent transition in which the temperature (Figure VII-4) and enthalpy (Figure VII-5) values are consistent with a combination of L_β/L_α and L_c/L_α phase transitions (Lewis and McElhaney, 1993). As a result, the overall enthalpy (Figure VII-5) of the cholesterol/DPPE mixture increases markedly at high cholesterol levels. Conversely, in the cooling mode, both the temperature (Figure VII-4) and enthalpy (Figure VII-5) of the chain-melting phase transition of cholesterol/DPPE mixtures consistently decrease with increasing cholesterol levels and by 50 mol% the transition is abolished. No other transitions were noted on cooling. Overall, it is clear that the thermotropic phase behavior of cholesterol/DPPE mixtures varies markedly from that documented in corresponding cholesterol/DPPC mixtures (McMullen *et al.*, 1993).

Shown in Figure VII-3 are the representative endotherms for the chain-melting phase transition of cholesterol/DSPE bilayers as a function of increasing cholesterol concentrations upon heating (left panel) and cooling (right panel). In the absence of cholesterol and in both the heating and cooling modes, DSPE bilayers exhibit an L_β to L_α phase transition at 74.3 °C with an enthalpy value of approximately 11.0 kcal/mol. When monitored in the heating mode, the addition of up to 10-15 mol% cholesterol to DSPE bilayers moderately decreases the temperature (Figure VII-4), enthalpy (Figure VII-5) and cooperativity (Figure VII-3) of the chain-melting phase transition. However, additional components are visible in the heating thermograms of cholesterol/DSPE mixtures containing 10 mol% or more cholesterol. As a result the enthalpy values from cholesterol/DSPE mixtures only moderately decrease with increasing cholesterol levels. Moreover, DSPE bilayers with 50 mol% cholesterol exhibit an endotherm with at least three large components with an overall enthalpy value (Figure VII-5) very close to that observed in pure DSPE bilayers. The transitions observed at approximately 57-60 °C and 69-73 °C in cholesterol/DSPE mixtures at cholesterol levels exceeding 20 mol% are consistent with the L_c/L_β and L_β/L_α phase transitions of pure DSPE bilayers, respectively (Lewis and McElhaney, 1993). When monitored in the cooling mode, the addition of up to 15 mol% cholesterol progressively decreases the temperature (Figure VII-4), enthalpy (Figure VIII-5) and cooperativity (Figure VII-3) of the L_α/L_β chain-melting transition. However, the endotherms of cholesterol/DSPE mixtures with cholesterol levels exceeding 15 mol% also exhibit multiple components which correspond to L_α/L_β and L_β/L_c transitions (Lewis and McElhaney, 1993). Consequently, even in the cooling mode, the overall enthalpy value (Figure VII-5) for the cholesterol/DSPE mixture does not reach zero by 50 mol% cholesterol. Thus, as in cholesterol/DMPE and cholesterol/DPPE mixtures, the thermotropic phase behavior and organization of cholesterol/DSPE mixtures does not resemble that documented for corresponding cholesterol/DSPC mixtures

(McMullen *et al.*, 1993), and the differences become increasing apparent with increasing cholesterol levels.

Effect of cholesterol on the thermotropic phase behavior and organization of DMPE, DPPE and DSPE bilayers: FTIR spectroscopic studies.

We performed FTIR experiments to determine the effect of cholesterol on the phase state and conformational disorder of the cholesterol/PE bilayers as a function of temperature and to correlate this information with the observed calorimetric transitions. The L_β/L_α or L_c/L_α phase transitions of the pure PEs lipids are accompanied by distinct changes in the CH_2 stretching, CH_2 scissoring and C=O stretching absorption bands which have been characterized in detail for the pure PEs by Lewis and McElhaney, (1993).

Shown in Figure VII-6 are representative FTIR spectra of the CH_2 stretching (left panel), the C=O stretching (middle panel), and CH_2 scissoring (right panel) absorption regions of aqueous dispersions of pure PE bilayers for the L_c1, L_c2, L_β and L_α phases. Note that the changes in the phase state and organization of the bilayers result in distinctive contours for each monitored band (see Lewis and McElhaney, 1993). Briefly, the CH_2 symmetric and asymmetric stretching band absorption maxima of PE bilayers are centered at 2848 and 2920 cm^{-1}, respectively, when the PE bilayer is in the L_c (type 1 or 2) or L_β phases (Figure VII-6, left panel). With increasing temperature the PE bilayers convert to the L_α phase and the CH_2 stretching absorption maxima of these bands shift up approximately 3 cm^{-1}. The C=O stretching band (Figure VII-6 middle panel) of the L_c1 phase of PE bilayers, the equilibrium crystalline phase, is a composite of two bands at 1744 and 1734 cm^{-1} while three distinct individual bands at approximately 1744, 1734 and 1724 cm^{-1} characterize the intermediate (L_c2) PE crystalline phase. The L_β phase exhibits a broad C=O stretching band and the underlying maxima shift to approximately 1740, 1728 and 1714 cm^{-1}. The C=O stretching band of L_α phase PE bilayers is considerably broader than the L_β phase, but the underlying components to the band are similar. The CH_2 scissoring bands of PE bilayers (Figure VII-6, right panel)., in either the L_c1 and L_c2 phase, exhibit band splitting with maxima at approximately 1471 and 1463 cm^{-1},while the L_β phase exhibits a single band at 1468 cm^{-1}. When the L_c1, L_c2 or L_β

phases convert to the L_α phase with increased temperature, the band absorption maxima broadens dramatically and shifts down to approximately 1468 cm^{-1} and a band representing CH_3 symmetric scissoring, at 1453-1456 cm^{-1}, becomes prominent. All of the changes observed in the monitored regions of the FTIR absorption spectrum of the PE bilayers correspond directly in temperature with the gel/gel and chain-melting phase transitions observed by HS-DSC.

The FTIR spectroscopic data of DMPE bilayers containing 5 to 30 mol% cholesterol, at low temperatures, are consistent with the L_β phase similar to that shown in Figure VII-6. With increasing temperature the cholesterol/DMPE mixtures (5 to 30 mol%) convert to the L_α phase with corresponding changes in the CH_2 stretching and scissoring bands and the C=O stretching band (see Figure VII-6). However, the DMPE bilayers containing 30 mol% and higher levels of cholesterol exhibit unique FTIR spectra depending on whether the spectra were obtained in the heating or cooling mode. Shown in Figure VII-7 are the representative C=O stretching (left panel) and CH_2 scissoring (right panel) bands for DMPE bilayers with 30-50 mol% cholesterol, for both heating and cooling scans as shown by arrows on the figure. At low temperatures the C=O stretching and CH_2 scissoring absorption band spectra for cholesterol/DMPE bilayers (Figure VII-7) represent a mixture of cholesterol-rich L_β PE and cholesterol-poor L_c2 PE phases which explains the difference between these absorption spectra and those shown for the pure PEs in Figure VII-6. The splitting of the CH_2 scissoring band observed above is particularly significant because it suggests that the PE hydrocarbon chains are packed in an orthorhombic perpendicular subcell (Snyder, 1961, 1979). Since the correlation field splitting can only occur when there are large domains of orthorhombically packed all-*trans* polymethylene chains, we conclude that large domains of virtually pure DMPE molecules must form even in the presence of high (>30 mol%) cholesterol concentrations. With increasing temperature, the C=O stretching and CH_2 scissoring bands of cholesterol/DMPE mixtures (>30 mol% cholesterol) convert directly to the L_α phase (Figure VII-7). Thus, the FTIR data confirms that the multiple components in the HS-DSC endotherms of cholesterol/DMPE mixtures (>30 mol% cholesterol) corresponds to overlapping L_c2/L_α and L_β/L_α phase transitions. Conversely, when monitored in the cooling mode, the CH_2 stretching, C=O stretching and CH_2 scissoring bands of cholesterol/DMPE mixtures (Figure VII-7) are consistent with the L_α phase at high temperature, which then converts directly to and L_β phase with decreasing temperature. Moreover, the temperature range of the L_α/L_β transition, when monitored by FTIR, was fully consistent with the phase transition observed in corresponding HS-DSC scans.

DPPE bilayers containing 0 to 10 mol% cholesterol appear to exhibit the spectroscopic signatures typical of an L_β/L_α phase transition, on both heating and cooling scans, as illustrated for the pure PEs in Figure VII-6. However, C=O stretching and CH_2 scissoring bands of cholesterol/DPPE mixtures containing >10 mol% cholesterol, at low temperatures, exhibit overlapping L_β-like and L_c2 phases very similar to that shown for cholesterol/DMPE bilayers in Figure VII-7. The small differences observed in either the C=O stretching or CH_2 scissoring absorption bands of the cholesterol/DPPE mixtures relative to pure PE bilayers are due to changes in the relative proportion of the cholesterol-rich L_β and the cholesterol-poor L_c2 PE domains in the cholesterol/DPPE mixtures. Thus, as in cholesterol/DMPE mixtures, cholesterol/DPPE mixtures at low temperature also must have extended arrays of cholesterol-poor L_c2 domains and cholesterol-rich L_β PE domains, even at cholesterol concentrations of 50 mol%. With increasing temperature, both the cholesterol-poor L_c2 and cholesterol-rich L_β phases convert to the L_α phase (Figure VII-7). On the other hand, in the cooling mode, the same cholesterol/DPPE mixtures (15 to 50 mol% cholesterol) exhibit broad L_β/L_α phase transitions with no indication of large arrays of cholesterol-poor PE domains (Figure VII-7). Thus, our FTIR spectroscopic data is fully consistent with the data from corresponding HS-DSC cooling scans of cholesterol/DPPE mixtures.

In all of the cholesterol/DSPE mixtures examined, the spectroscopic data is consistent with partially overlapping L_c1/L_β and L_β/L_α phase transitions on heating (Figure VII-8), which correlates with the HS-DSC scans of corresponding mixtures. The only difference between the different cholesterol/DSPE mixtures is the increasing proportion of the cholesterol-poor L_c1 relative to the cholesterol-rich L_β PE phase domains in the mixture with decreasing temperature and increasing cholesterol levels (results not shown). It is important to note that the formation of a crystalline phase in DSPE bilayers has not been documented before in the pure fully hydrated system, thus it is remarkable that it should occur, even partially, in bilayers containing significant amounts of cholesterol (see Lewis and McElhaney, 1993). Moreover, the crystalline phase of the cholesterol/DSPE bilayers closely resembles the stable L_c1 phase, unlike the metastable L_c2 phase observed in cholesterol/DMPE and cholesterol/DPPE mixtures. Thus the lateral phase separation of cholesterol and DSPE in cholesterol/DSPE mixtures is likely on a larger scale than that observed for corresponding cholesterol/DMPE and cholesterol/DPPE mixtures. In the cooling mode, the. L_α phase of cholesterol/DSPE bilayers, which closely resembles the L_α phase of cholesterol/DMPE and cholesterol/DPPE bilayers, converts to the L_β phase just below the phase transition temperature (Figure VII-8). With further decreases in temperature the L_β-like phase converts to the L_c1 phase, fully consistent with our HS-DSC cooling scans of cholesterol/DSPE mixtures with > 20 mol% cholesterol.

By combining HS-DSC and FTIR spectroscopic data we have also determined that the thermotropic phase behavior and organization of cholesterol/PE mixtures depends on the thermal history of the sample and the scan rate of the HS-DSC experiments. Shown in Figure VII-9 are DSC thermograms and the CH_2 asymmetric absorption maxima of DMPE (left panel), DPPE (middle panel) and DSPE (right panel) bilayers with 35 mol% cholesterol, after allowing the samples to equilibrate at low temperature (2 °C) for 1 week. The HS-DSC thermograms were done at the lowest possible scan rate (5 °C/hr) in order to minimize kinetic effects and maximize their comparability to the spectroscopic data. Over the temperature range of the PE hydrocarbon chain-melting endotherms observed by HS-DSC (bottom of Figure VII-9), there are corresponding increases in the conformational disorder of the hydrocarbon chains as indicated by the upshift in the frequency of the CH_2 asymmetric stretching band maxima (top of Figure VII-9). In each of the cholesterol/PE mixtures, the size of the main chain-melting endotherm(s) (and the enthalpy) has increased markedly compared to that shown for corresponding cholesterol/PE samples which were equilibrated for 1 day and scanned at 10 °C/hr (Figures VII-1, -2 and -3). Thus, the relative proportion of the cholesterol-poor L_c phase domains increases at the expense of the cholesterol-rich L_β phase DMPE phospholipid domains when equilibrated at progressively longer times at low temperatures. Moreover, changes in the relative proportions of the cholesterol-rich L_β PE and cholesterol-poor L_c PE phases in cholesterol/PE mixtures at low temperatures explains the complex calorimetric and spectroscopic data observed with increasing cholesterol levels. We also observe additional low temperature endotherms in cholesterol/DPPE and cholesterol/DSPE mixtures that do not correspond to chain-melting transitions, as indicated by the lack of a significant shift in the absorption maxima of the CH_2 stretching bands (top Figure VII-9), or gel state polymorphism of pure PE bilayers (Lewis and McElhaney, 1993). Furthermore, these additional endotherms are not characteristic of the anhydrous cholesterol polymorphic transition, nor the cholesterol monohydrate/anhydrous phase transition at 86 °C (see Loomis *et al.*, 1979). However, the present spectroscopic and calorimetric evidence does suggest that the additional endotherms represent a temperature-induced cooperative lateral dissolution of cholesterol in gel-state PE bilayers similar to that previously documented for androstenol/PC mixtures by this laboratory (McMullen *et al.*, 1994). Thus, with progressively slower scan rates and and longer low temperature incubations times, the more pronounced the lateral phase separation of cholesterol in the host PE bilayer.

Effect of cholesterol on the thermotropic phase behavior and organization of DMPE, DPPE and DSPE bilayers: ^{31}P-NMR Spectroscopic studies.

We employed ^{31}P-NMR to monitor the effect of cholesterol on the mobility of the PE headgroups as well as to confirm that the additional endotherms observed in the various cholesterol/PE bilayers represent transitions between lamellar phases. For reference, the L_c (type 1 or 2), L_β and L_α phases of pure PE bilayers are shown in Figure VII-10. In both of the L_β and L_α phases, all of the lipids exhibit axially symmetric powder patterns that are indicative of axially symmetric motion of the phosphodiester headgroup on the surface of a lipid bilayer. Note that when the L_c phase forms, the T1 relaxation times of the rigid phosphorous nuclei in this highly ordered and immobile crystalline lattice are so slow that virtually no signal is observed (Lewis and McElhaney, 1993), in contrast to the L_β or L_α phases.

On stepwise heating for cholesterol concentrations >30 mol% in cholesterol/DMPE (Figure VII-11), >10-15 mol% in cholesterol/DPPE (Figure VII-11) and >5 mol% in cholesterol/DSPE mixtures (Figure VII-12), the ^{31}P-NMR spectra are consistent with a mixture of cholesterol-rich L_β and cholesterol-poor L_c (type 1 or 2) PE phases at low temperatures (see Figure VII-11). With increasing temperature, all of the cholesterol/PE mixtures will convert to the L_α phase directly (DMPE and DPPE, Figure VII-11) or via the L_β phase (DSPE, Figure VII-12). Conversely, when monitored on cooling, cholesterol/DMPE and cholesterol/DPPE mixtures containing 0 to 50 mol% cholesterol exhibit spectra characteristic of an L_α to L_β phase transition (Figure VII-10). For cholesterol/DSPE mixtures containing 20 to 50 mol% cholesterol when monitored on cooling, we observe spectra characteristic of an L_α/L_β transition followed by a L_β/L_c1 phase transition with decreasing temperature (Figure VII-12). Overall, our ^{31}P-NMR data correspond well with our HS-DSC and FTIR data, confirming that cholesterol-rich and cholesterol-poor domains exist in PE bilayers, even in cholesterol/PE samples containing as much as 50 mol% cholesterol.

DISCUSSION

In a prior study, we (McMullen *et al.*, 1993) found that the incorporation of increasing levels of cholesterol into saturated PC bilayers with hydrocarbon chain lengths of 13:0 to 21:0 carbons progressively decreased the cooperativity and enthalpy of the chain melting transition of the host PC bilayer, in both the heating and cooling modes, such that by 50 mol% cholesterol the chain-melting phase transition was completely abolished. Thus, cholesterol appears to be fully miscible in both the gel and liquid-crystalline states of the host PC bilayer, regardless of PC hydrocarbon chain length, and the stoichiometry of cholesterol-PC interactions is constant at approximately 1 cholesterol molecule per 3.5 PC molecules (Engleman and Rothman, 1972; Mabrey *et al.*, 1978; Estep *et al.*, 1978; McMullen *et al.*, 1993). The homogenous distribution of cholesterol in DPPC bilayers has also been determined directly by studies using fluorescent probes (Tang *et al.*, 1994; Chong, 1995). The only chain length-dependent effect of observed in cholesterol/PC mixtures is the relative destabilization (stabilization) of the PC gel state upon cholesterol incorporation into PC bilayers with hydrocarbon chains longer (shorter) than 17:0 PC (McMullen *et al.*, 1993; Chia *et al.*, 1993). This effect was attributed to the hydrophobic mismatch effect first described by Mouritsen and Bloom (1984) for transmembrane peptides in PC bilayers of varying hydrophobic thickness. However, in this study, we find that the thermotropic phase behavior and organization of cholesterol/PE mixtures of varying hydrocarbon chain length and cholesterol concentrations differs markedly from that documented for cholesterol/PC mixtures, as well as from prior studies of cholesterol/PE mixtures (van Dijck *et al.*, 1976; Demel *et al.*, 1977; Blume, 1980; Blume, 1982). Specifically, we demonstrate that the stoichiometry of cholesterol-PE interactions is considerably lower in the gel than the liquid-crystalline state of PE bilayers. Moreover, the stoichiometry of cholesterol-PE interactions also depends on the hydrocarbon chain length of the host PE bilayer as well as the level of cholesterol and the thermal history of the sample. Finally, we do not observe hydrophobic mismatch-induced changes in the relative stability of the gel and liquid-crystalline states of cholesterol/PE mixtures with varying PE hydrocarbon chain lengths. We attribute the unique thermotropic phase behavior and organization of cholesterol/PE mixtures to the relatively poor miscibility of cholesterol in PE relative to PC bilayers, particularly at low temperatures and high cholesterol levels. The limited miscibility of cholesterol in PE relative to PC bilayers is probably due to the relatively strong inter-headgroup hydrogen bonding and electrostatic interactions of the former phospholipid, which favors phospholipid-phospholipid contacts over cholesterol-phospholipid interactions (Blume, 1980; Boggs, 1987). Thus, at low temperatures, where the tight packing of neighboring PE molecules permits extensive non-polar and polar intermolecular interactions, the intercalation of cholesterol between neighboring PEs is highly unfavorable, and becomes more so with increasing cholesterol levels. As a result, the number of cholesterol-PE contacts, and thus the effective cholesterol-PE interaction stoichiometry, is considerably lower than in corresponding cholesterol/PC mixtures. This allows for the formation of extended arrays of ordered, crystalline PE domains in cholesterol/PE mixtures from which cholesterol must be completely excluded (Lewis and McElhaney, 1993). This explains why we observe coexisting domains of cholesterol-rich L_β and cholesterol-poor L_c phases in all of the cholesterol/PE mixtures examined, even up to 50 mol% cholesterol. Moreover, the magnitude, and the rate, of the lateral phase separation of cholesterol-rich L_β and cholesterol-poor L_c (type 1 or 2) phases in cholesterol/PE mixtures increase with increasing PE hydrocarbon chain length. This is due to the increased van der Waals forces between the hydrocarbon chains of neighboring PE molecules which further favors phospholipid-phospholipid contacts over cholesterol-phospholipid interactions. The temperature- and cholesterol concentration-dependent calorimetric and spectroscopic data which we documented in cholesterol/PE mixtures can thus be explained as a consequence of variations in the relative proportions of coexisting cholesterol-rich gel and cholesterol-poor crystalline PE domains. However, as the temperature increases and progresses beyond both the L_β/L_α or L_c/L_α chain-melting phase transitions and the strength of the PE attractive intermolecular forces decreases, the lateral exclusion of cholesterol becomes less favorable, and progressively larger amounts of cholesterol dissolve into the host PE bilayer. This accounts for the substantially enhanced effect of cholesterol on the enthalpy, cooperativity and temperature of the PE chain-melting phase transition observed upon subsequent cooling of a given cholesterol/PE sample, such that the thermotropic phase behavior of the cholesterol/PE system more closely resembles corresponding cholesterol/PC mixtures. However, our calorimetric and spectroscopic studies of cholesterol/DSPE mixtures reveal that cholesterol levels above 50 mol% are not sufficient to abolish the chain-melting transition on either heating or cooling. Thus, in contrast to the shorter chain cholesterol/PE mixtures or corresponding longer chain cholesterol/PC mixtures, the apparent stoichiometry of cholesterol-PE interactions is limited even in the relatively disordered state of liquid-crystalline DSPE bilayers. In fact, when we attempted to study cholesterol/PE mixtures where the hydrocarbon chain length of the PE was 20 carbons, we found it extremely difficult to form stable mixtures due to the very low miscibility of cholesterol both gel and liquid-crystalline state DAPE bilayers. Thus it appears that both PE-cholesterol hydrophobic mismatch and the strong PE headgroup interactions contribute to the relative immiscibility of cholesterol in PE as compared to PC bilayers. In summary, we demonstrate for the first time that the miscibility of cholesterol, and thus the effective stoichiometry of cholesterol-PE interactions, in PE bilayers varies with hydrocarbon chain length, temperature and cholesterol concentration. This conclusion is in marked contrast to those of prior studies of cholesterol/PE mixtures, which assume static cholesterol-phospholipid interaction stoichiometries to account for the effect of cholesterol on the thermotropic phase behavior and organization of the host bilayer (van Dijck *et al.*, 1976; Demel *et al.*, 1977; Cullis *et al.*, 1978; Blume, 1980; Blume and Griffin, 1982; Epand and Bottega, 1987; Cheetham *et al.*, 1989).

One of the most surprising findings in this investigation was the observation of cholesterol-poor, highly ordered crystalline (type 1 or 2) phases in cholesterol/PE mixtures, especially at cholesterol concentrations as high as 50 mol%. Moreover, cholesterol appears to facilitate the formation of PE L_c (type 1 and 2) phases, as cholesterol-containing PE bilayers form the L_c phases much faster than PE bilayers without cholesterol. While this result is in marked contrast to the present understanding of the effect of cholesterol on the thermotropic phase behavior and organization of PC's, it is important to note that qualitatively similar results were observed in PE bilayers containing α-helical transmembrane peptides (Zhang *et al.*, 1995a). The mechanism that Zhang *et al.* (1995a) propose to rationalize the formation of peptide-poor crystalline PE domains in peptide/PE mixtures is based on the lateral phase-separation of peptide and PE. The peptide-rich, PE-poor domain provides a large hydrophobic surface which nucleates the formation of the dehydrated, tightly packed L_c (type 1 or 2) phase in PE molecules at the domain interface. Similarly, the lateral phase separation of cholesterol and PE, with subsequent formation of poorly hydrated domains of cholesterol, may nucleate the formation of the highly ordered, cholesterol-poor crystalline phase at the domain interface. This accounts for the coexisting cholesterol-rich L_β and cholesterol-poor L_c phases in cholesterol/PE mixtures, as well as the increasingly rapid formation of crystalline phases with increasing cholesterol levels.

In order to determine the "affinity" of cholesterol for different phospholipid species, in particular for PC's and PE's, prior DSC studies used monotectic mixtures invariably contained a combination of saturated and unsaturated phospholipids with varying hydrocarbon chain lengths (van Dijck *et al.*, 1976, 1979; Demel *et al.*, 1977; Calhoun and Shipley, 1979). However, it is now clear that the hydrocarbon chain composition, the level of cholesterol and the scan rate all have a considerable influence on the thermotropic phase behavior and organization of the cholesterol/PE mixtures, in marked contrast to chain length-independent miscibility of cholesterol in corresponding PC bilayers (McMullen *et al.*, 1993, 1995; Ali *et al.*, 1994; Vilchèze *et al.*, 1996). Thus, the apparent affinity of cholesterol for different phospholipids will vary with scan rate and the hydrocarbon chain length(s) of the host phospholipids. Moreover, the distribution of cholesterol in higher order cholesterol/phospholipid mixtures will depend on whether or not the presence of cholesterol will inhibit, (as in the case of the PCs) or promote (as in the case of the PEs) the ability of the phospholipid species to form crystalline phases. This may account for the discrepant results of prior cholesterol/DMPE and cholesterol/DPPE HS-DSC and cholesterol/DPPE ^{13}C- and ^{2}H-NMR studies (Blume, 1980; Blume and Griffin, 1982). The NMR experiments involved holding the cholesterol/DPPE mixtures at a given temperature for considerable periods of time, while the corresponding cholesterol/DMPE HS-DSC experiments involved scan rates of no less than 60 °C/hr. As a result, NMR experiments favor the formation of laterally phase separated cholesterol-rich L_c and cholesterol-poor L_β phases in cholesterol-DPPE mixtures, especially at low temperatures and high cholesterol levels. In fact, the authors observe metastability in that region of their temperature/composition plot, while HS-DSC experiments identified only the L_β and L_α phases of the shorter chain cholesterol/DMPE mixtures. Thus, the present work demonstrates that a multidisciplinary approach is required to systematically characterize the thermotropic phase behavior and organization of binary and higher order cholesterol-containing phospholipid mixtures.

We have demonstrated that the distribution of cholesterol within PE bilayers, and the subsequent effect of cholesterol on the thermotropic phase behavior and organization of the host bilayer, varies with hydrocarbon chain length and temperature. When these results are considered with those of corresponding PC/cholesterol mixtures (McMullen *et al.*, 1993), is clear that the differential affinity of cholesterol for different phospholipid bilayers depends on both the length of the phospholipid hydrocarbon chains and the ability of the phospholipid headgroups to participate in attractive inter-headgroup hydrogen bonding and electrostatic interactions. Interestingly, the same factors appear to govern the miscibility of transmembrane peptides with PC and PE bilayers (Zhang *et al.*, 1992a,b, 1995b,c). Thus the differential affinity of cholesterol, or transmembrane peptides, for different phospholipids may form the basis for the formation of domains of phospholipids selectively enriched (PC) or depleted (PE) in cholesterol and/or transmembrane peptides. We note that differences in the size and morphology of cholesterol-rich and -poor domains as a function of temperature and phospholipid composition has been directly observed in cholesterol/SPM and cholesterol/PC mixtures of varying hydrocarbon chain lengths (Chong, 1994; Tang *et al.*, 1995; Mattjus *et al.*, 1995; Slotte and Mattjus, 1995). We predict that investigations using magic angle spinning-NMR or fluorescent spectroscopy will demonstrate that the lateral organization of cholesterol in PE bilayers also differs significantly from cholesterol/PC (or cholesterol/SPM) mixtures and will also vary with temperature and the level of cholesterol present (see Guo and Hamilton, 1995; Chong, 1994; Mattjus *et al.*, 1995; Slotte and Mattjus, 1995; Tang *et al.*, 1995). Moreover, the homogeneous and temperature-independent distribution of cholesterol within the bilayer of eukaryotic cell plasma membranes sometimes assumed, based on the properties of the so-called liquid-ordered phase of PC bilayers containing high levels of cholesterol (Ipsen *et al.*, 1987; Vist and Davis, 1990; Thewalt and Bloom, 1992), may be an oversimplification since we have shown that that this is clearly not the case for cholesterol/PE mixtures (McMullen *et al.*, 1996). Thus the dynamic and heterogeneous organization of cholesterol-containing phospholipid bilayers with appreciable levels of PE, such as eukaryotic cell plasma membranes, could have a considerable impact on the organization of the lipid phase of biological membranes and the formation of domains based on selective phospholipid-phospholipid, cholesterol-phospholipid or protein-cholesterol interactions (Thompson *et al.*, 1992; Glaser, 1993; Welti and Glaser, 1994; Kinnunen *et al.*, 1996; Vaz and Alameida, 1993).

Figure VII-1. Representative heating (left panel) and cooling (right panel) thermograms of DMPE bilayers containing progressively increasing levels of cholesterol as indicated on the Figure. HS-DSC scans of DMPE bilayers containing 25 and 30 mol% cholesterol were also performed but are not shown. All endotherms shown were scanned at 10 °C/hr and are normalized for sample mass.

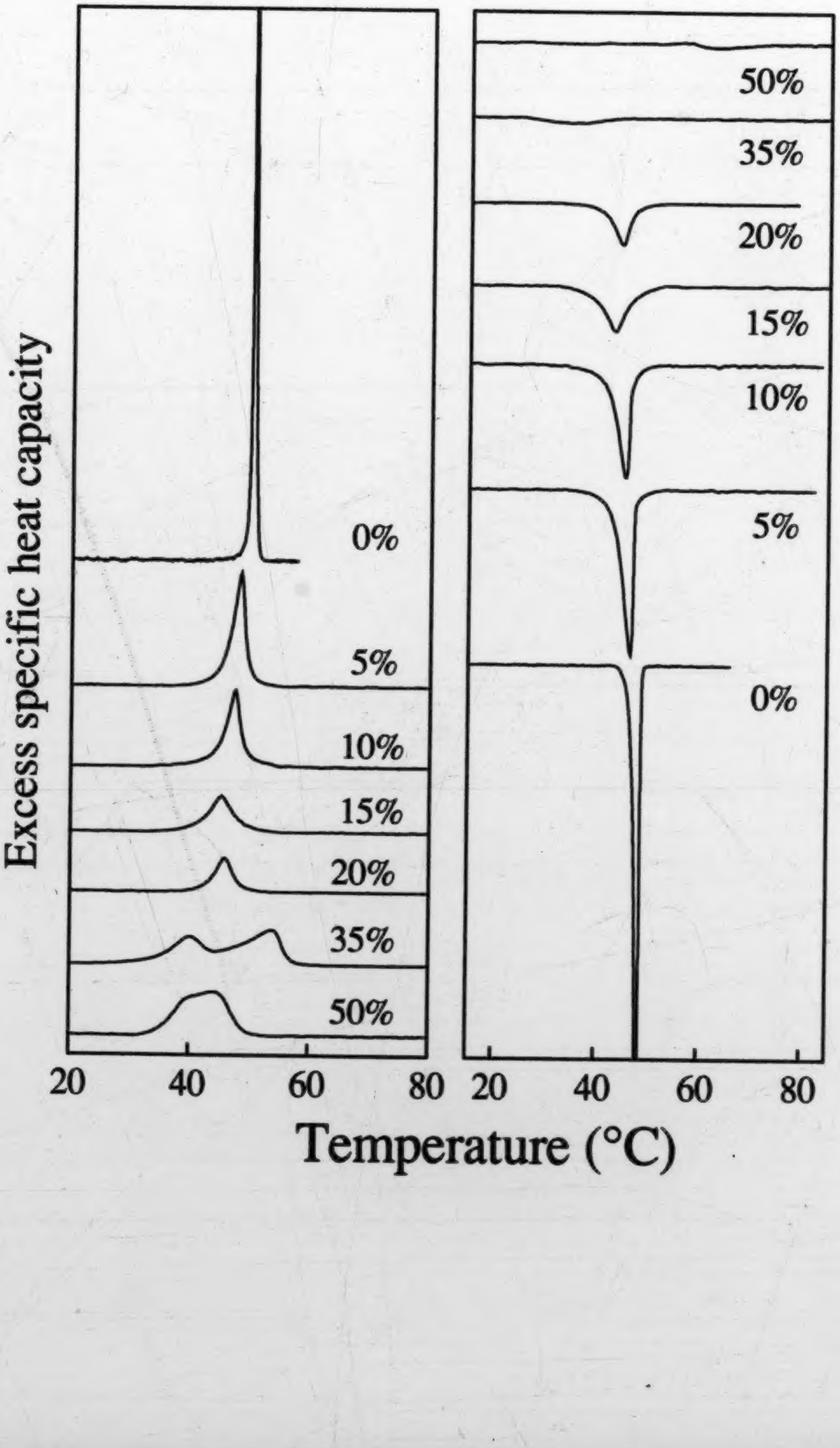

Excess specific heat capacity
0%
5%
10%
15%
20%
35%
50%
50%
35%
20%
15%
10%
5%
0%
20
40
60
80
20
40
60
80
Temperature (°C)

Figure VII-2. Representative heating (left panel) and cooling (right panel) thermograms of DPPE bilayers containing progressively increasing levels of cholesterol as indicated on the Figure. HS-DSC scans of DPPE bilayers containing 25 and 30 mol% cholesterol were also performed but are not shown. All endotherms shown were scanned at 10 °C/hr and are normalized for sample mass.

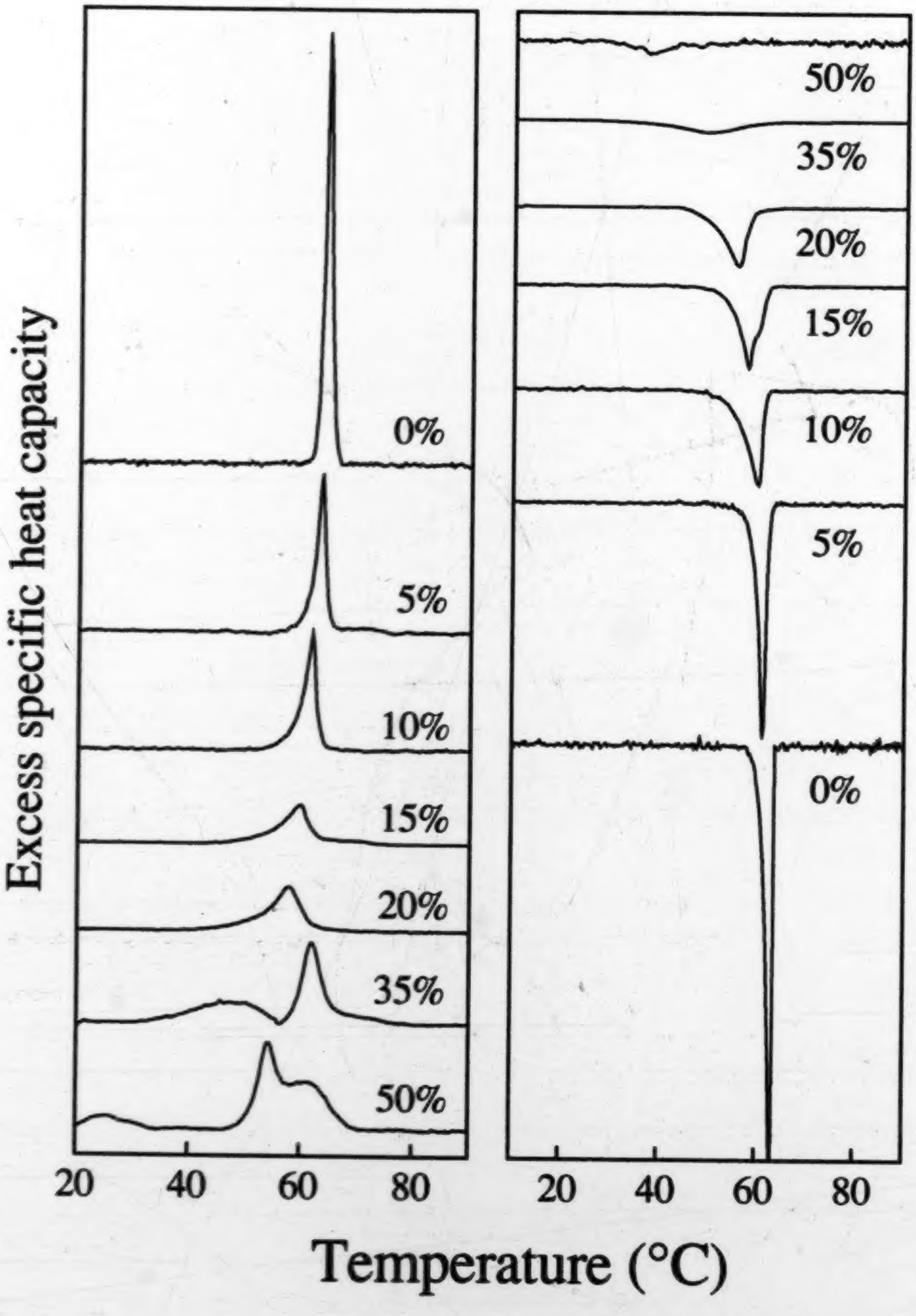

Excess specific heat capacity
0%
5%
10%
15%
20%
35%
50%
20
40
60
80
50%
35%
20%
15%
10%
5%
0%
20
40
60
80
Temperature (°C)

Figure VII-3. Representative heating (left panel) and cooling (right panel) endotherms of DSPE bilayers containing progressively increasing levels of cholesterol as indicated on the Figure. HS-DSC scans of DSPE bilayers containing 25 and 30 mol% cholesterol were also performed but are not shown. All endotherms shown were scanned at 10 °C/hr and are normalized for sample mass.

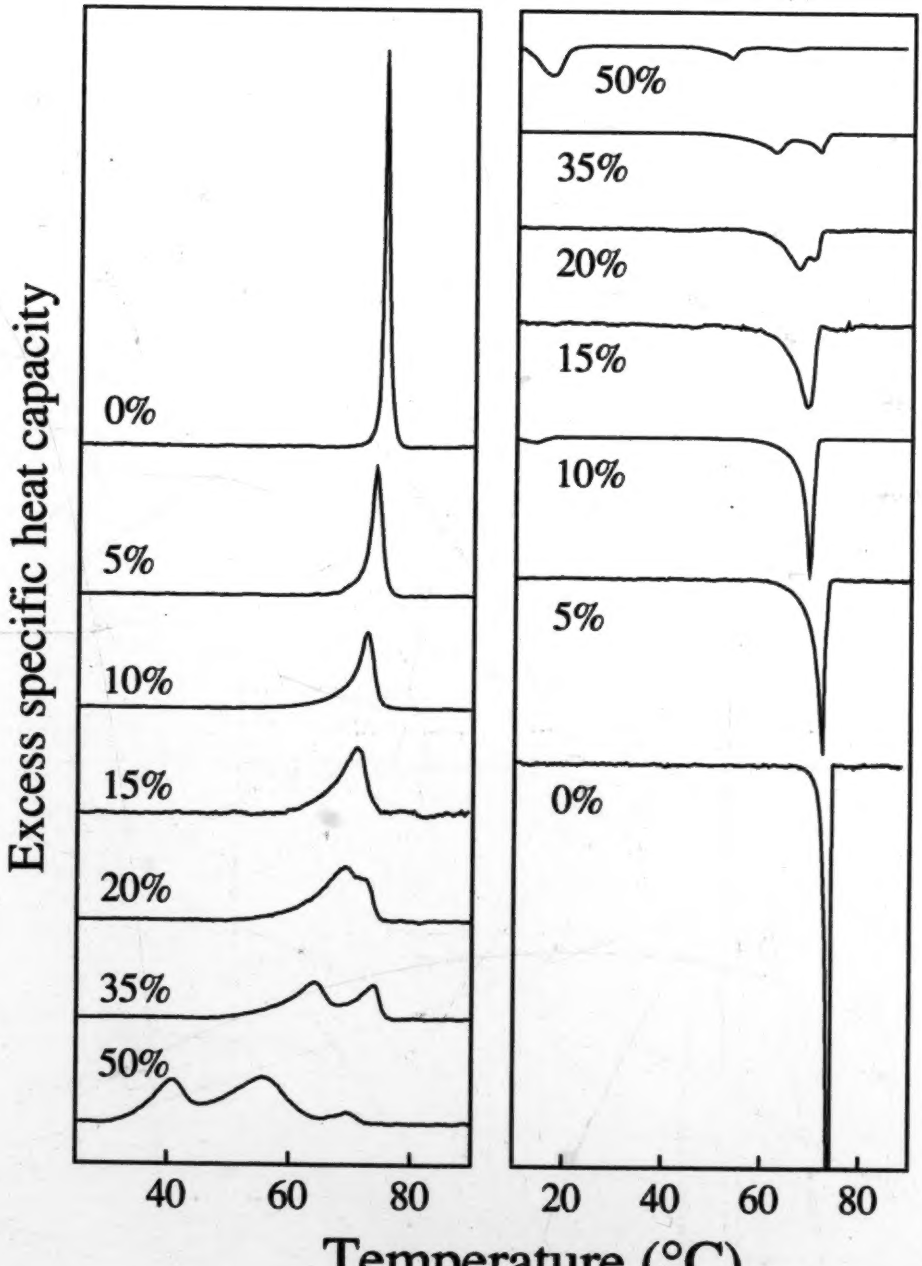

Excess specific heat capacity
0%
5%
10%
15%
20%
35%
50%
40
60
80
50%
35%
20%
15%
10%
5%
0%
20
40
60
80
Temperature (°C)

Figure VII-4. Plots of the transition temperature of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DMPE, cholesterol/DPPE and cholesterol/DPPE mixtures obtained from both heating and cooling runs. Transition temperature values obtained upon heating are represented by solid symbols and upon cooling by open symbols. The dotted lines represent the midpoint temperatures of additional components (L_c/L_β or L_c/L_α) in cholesterol/PE mixtures. Symbols used are: cholesterol/DMPE mixtures (■,□); cholesterol/DPPE mixtures (●,○); cholesterol/DSPE mixtures (◆,◊). All points are averaged over at least three separate determinations, all values +/- 0.5 °C.

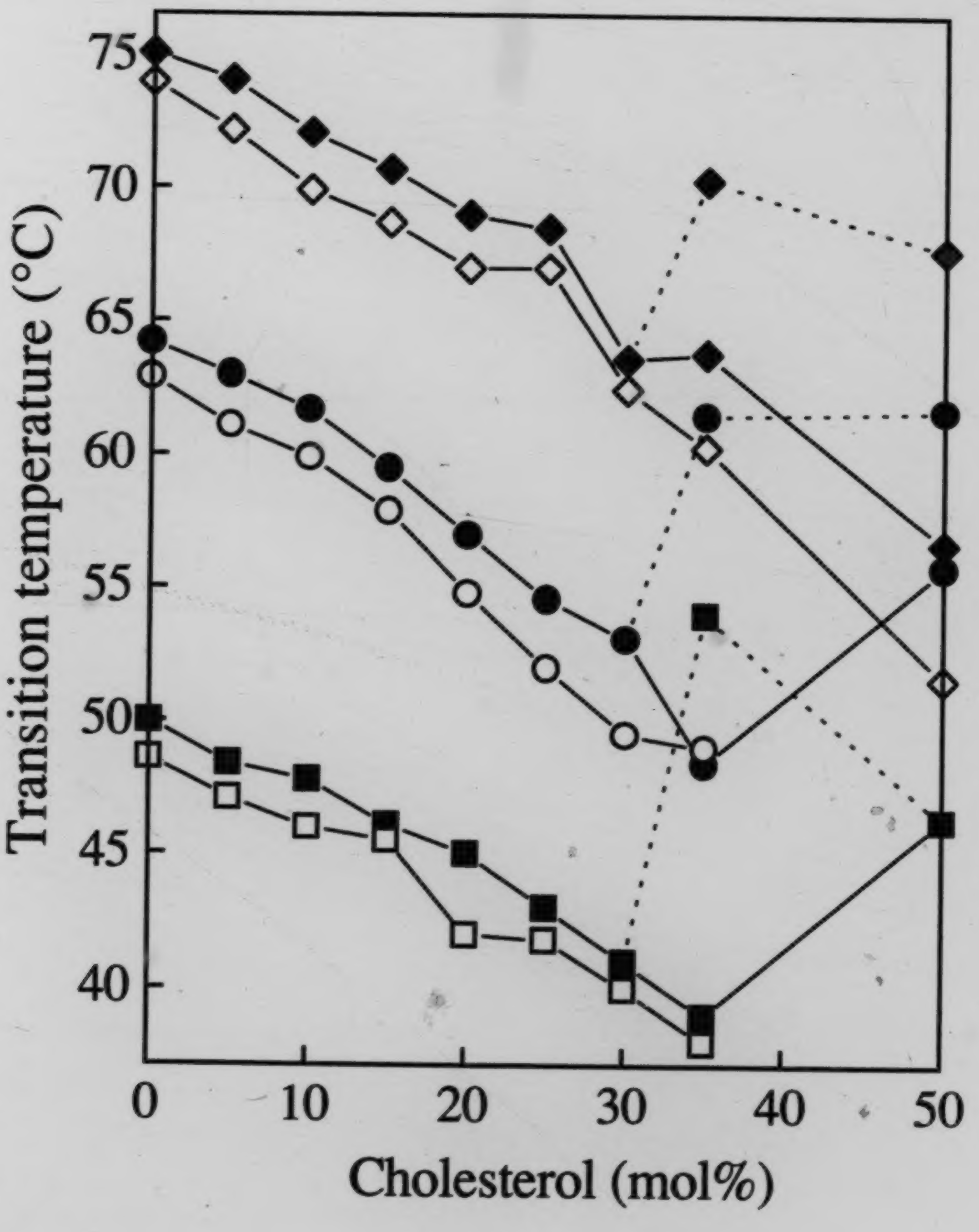
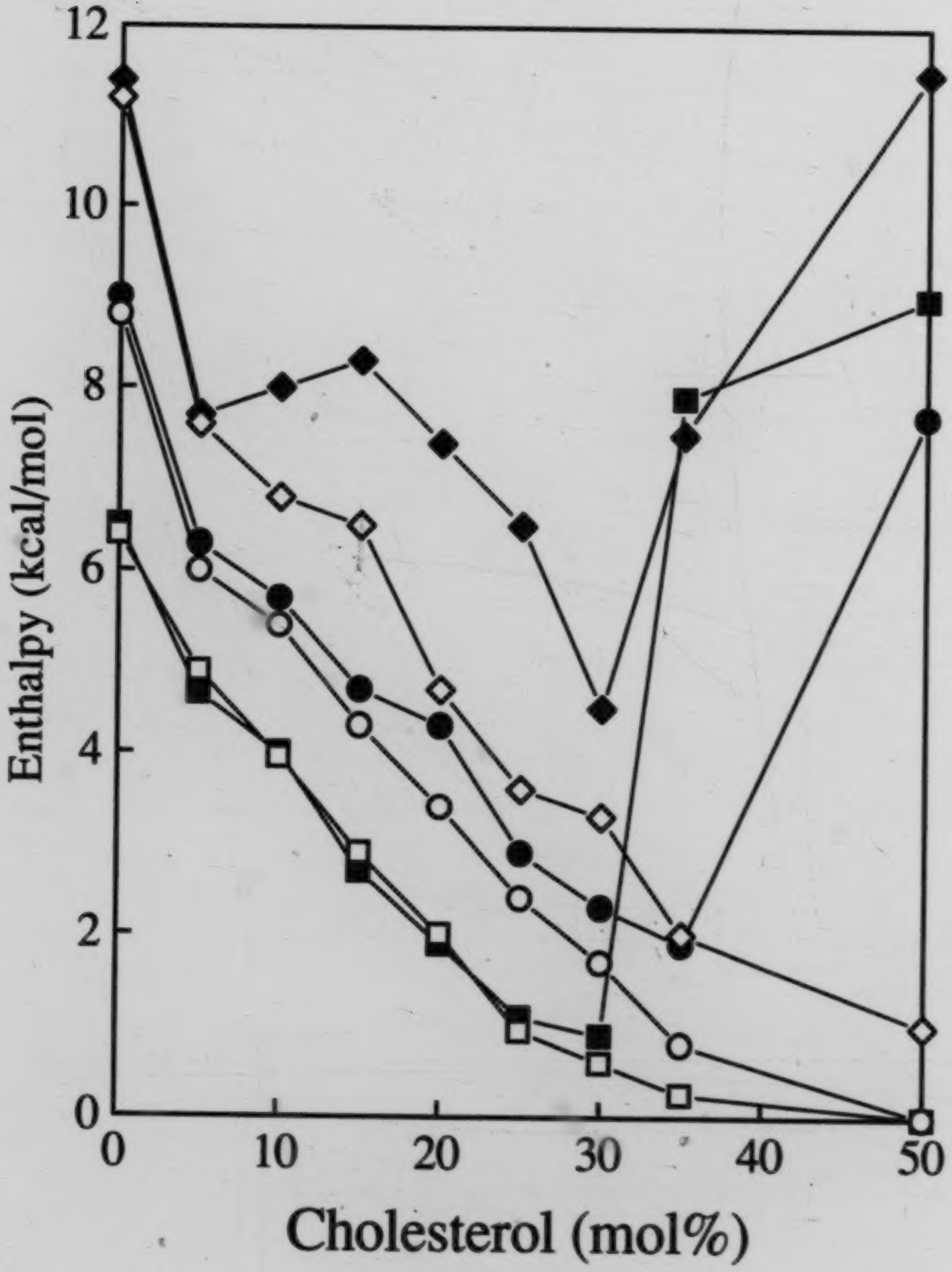

Transition temperature (°C)
75
70
65
60
55
50
45
40
0
10
20
30
40
50
Cholesterol (mol%)

Figure VII-5. Plots of the overall enthalpy of the chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DMPE, cholesterol/DPPE and cholesterol/DSPE mixtures obtained from both heating and cooling runs. Enthalpy values obtained upon heating are represented by solid symbols and upon cooling by open symbols. Symbols used are: cholesterol/DMPE mixtures (■,□); cholesterol/DPPE mixtures (●,○); cholesterol/DSPE mixtures (◆,◊). All points are averaged over at least three separate determinations, all values +/- 0.30 kcal/mol.



12
10
8
6
4
2
0
Enthalpy (kcal/mol)
0
10
20
30
40
50
Cholesterol (mol%)

Figure VII-6. Representative FTIR CH_2 stretching, C=O stretching and CH_2 scissoring absorption bands for the L_α, L_β, L_c1 and L_c2 phases of fully hydrated n-saturated diacyl PE bilayers, as indicated in the Figure. The left panel represents the CH_2 symmetric and asymmetric stretching bands, the middle panel the C=O stretching absorption bands and the right panel the CH_2 scissoring absorption bands.

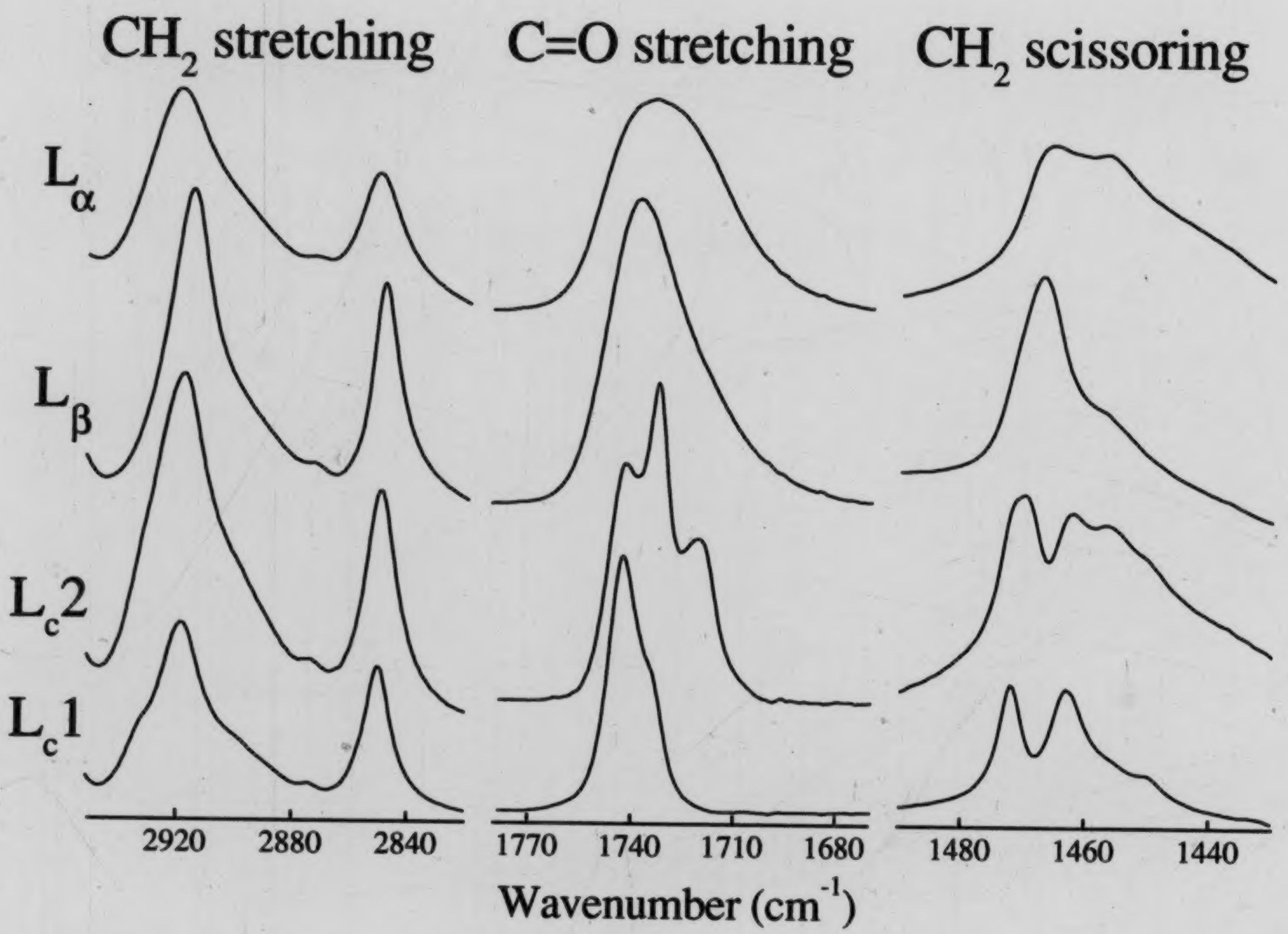

CH2 stretching
C=O stretching
CH2 scissoring
Lα
Lβ
Lc2
Lc1
2920
2880
2840
1770
1740
1710
1680
1480
1460
1440
Wavenumber (cm-1)

Figure VII-7. Representative FTIR C=O stretching and CH_2 scissoring absorption bands for DMPE bilayers containing >30 mol% cholesterol and DPPE bilayers containing >10 mol% cholesterol (see text for details). Shown in the left panel are the C=O stretching absorption bands and in the right panel the CH_2 scissoring absorption bands. Arrows indicate phase changes with increasing (solid) or decreasing (dotted) temperature.

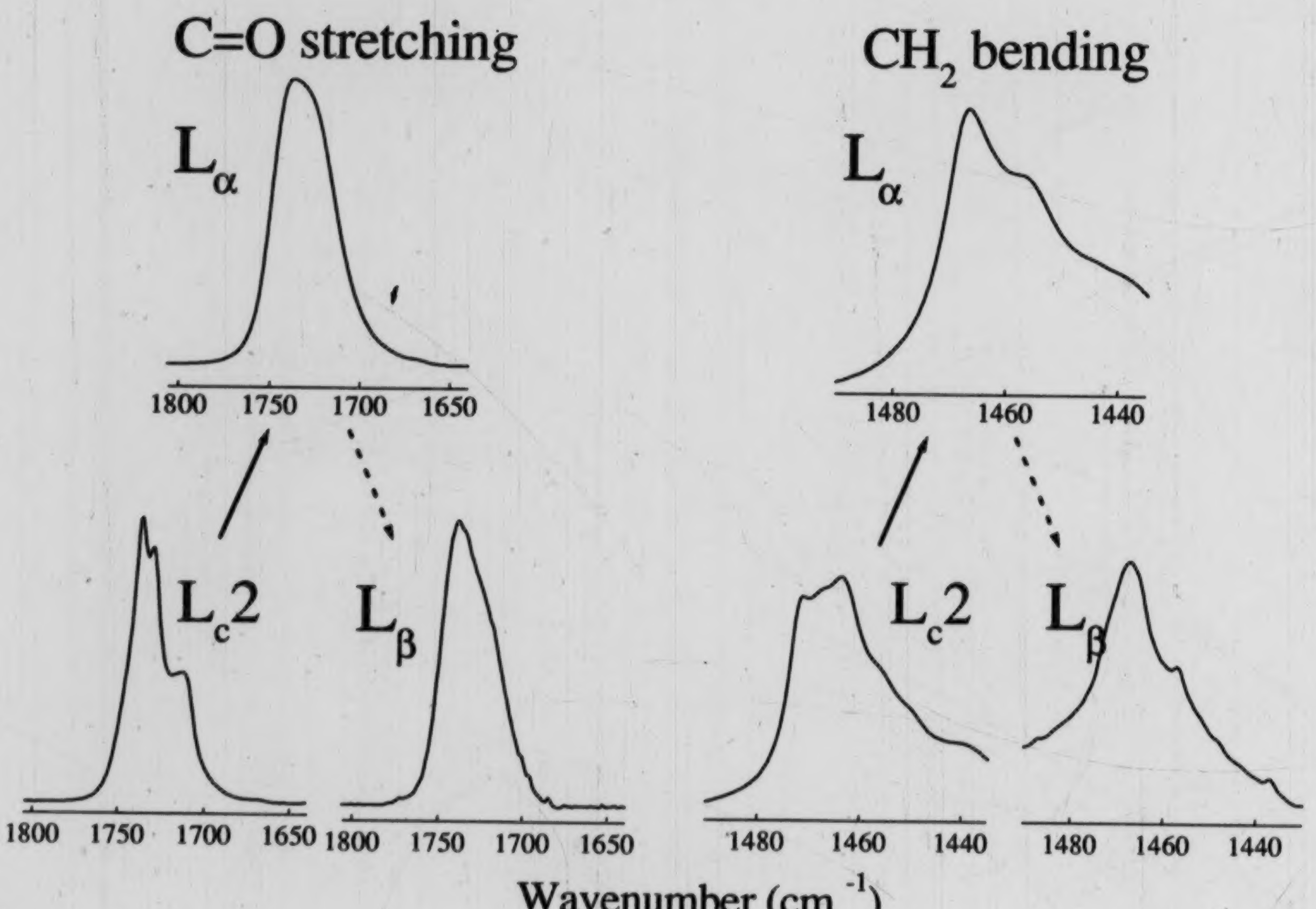

C=O stretching
CH$_2$ bending
L$_\alpha$
L$_\alpha$
L$_c$2
L$_\beta$
L$_c$2
L$_\beta$
1800
1750
1700
1650
1480
1460
1440
Wavenumber (cm^{-1})

Figure VII-8. Representative FTIR C=O stretching and CH_2 scissoring absorption bands for DSPE bilayers containing various levels of cholesterol (see text for details). Shown in the left panel are the C=O stretching absorption bands and in the right panel the CH_2 scissoring absorption bands. Arrows indicate phase changes with increasing (solid) or decreasing (dotted) temperature.

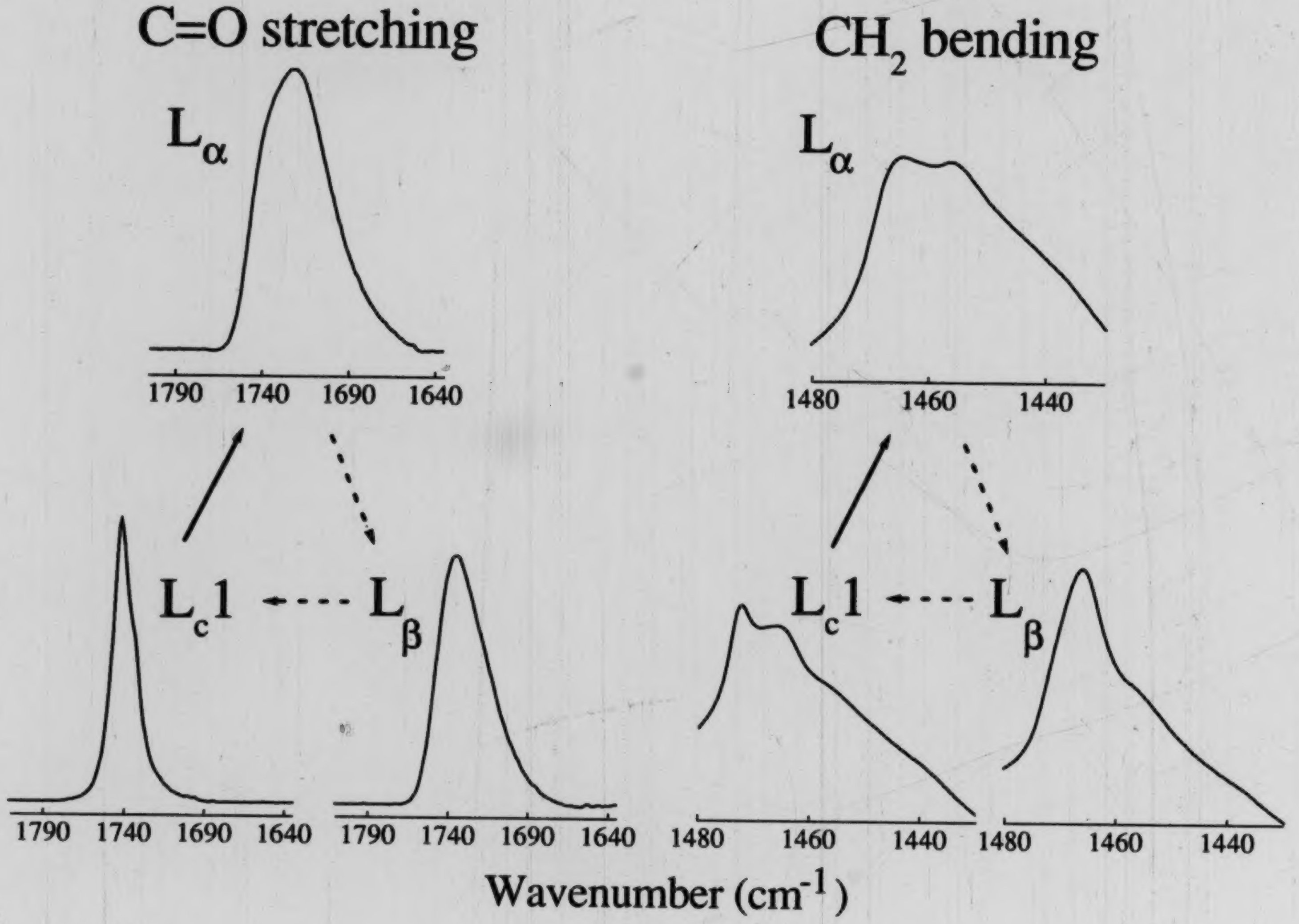
C=O stretching
CH$_2$ bending
L$_\alpha$
L$_\alpha$
L$_c$1
L$_\beta$
L$_c$1
L$_\beta$
1790
1740
1690
1640
1480
1460
1440
Wavenumber (cm^{-1})

Figure VII-9. A comparative plot of the HS-DSC thermograms (scanned in the heating mode at 5 °C/hr), across bottom of Figure, of DMPE (left panel), DPPE (middle panel) and DSPE (right panel) bilayers each containing 35 mol% cholesterol with the corresponding absorption maxima of the CH_2 symmetric stretching region of the FTIR spectrum (top). See text for detailed description of phase behavior of each cholesterol/PE mixture.

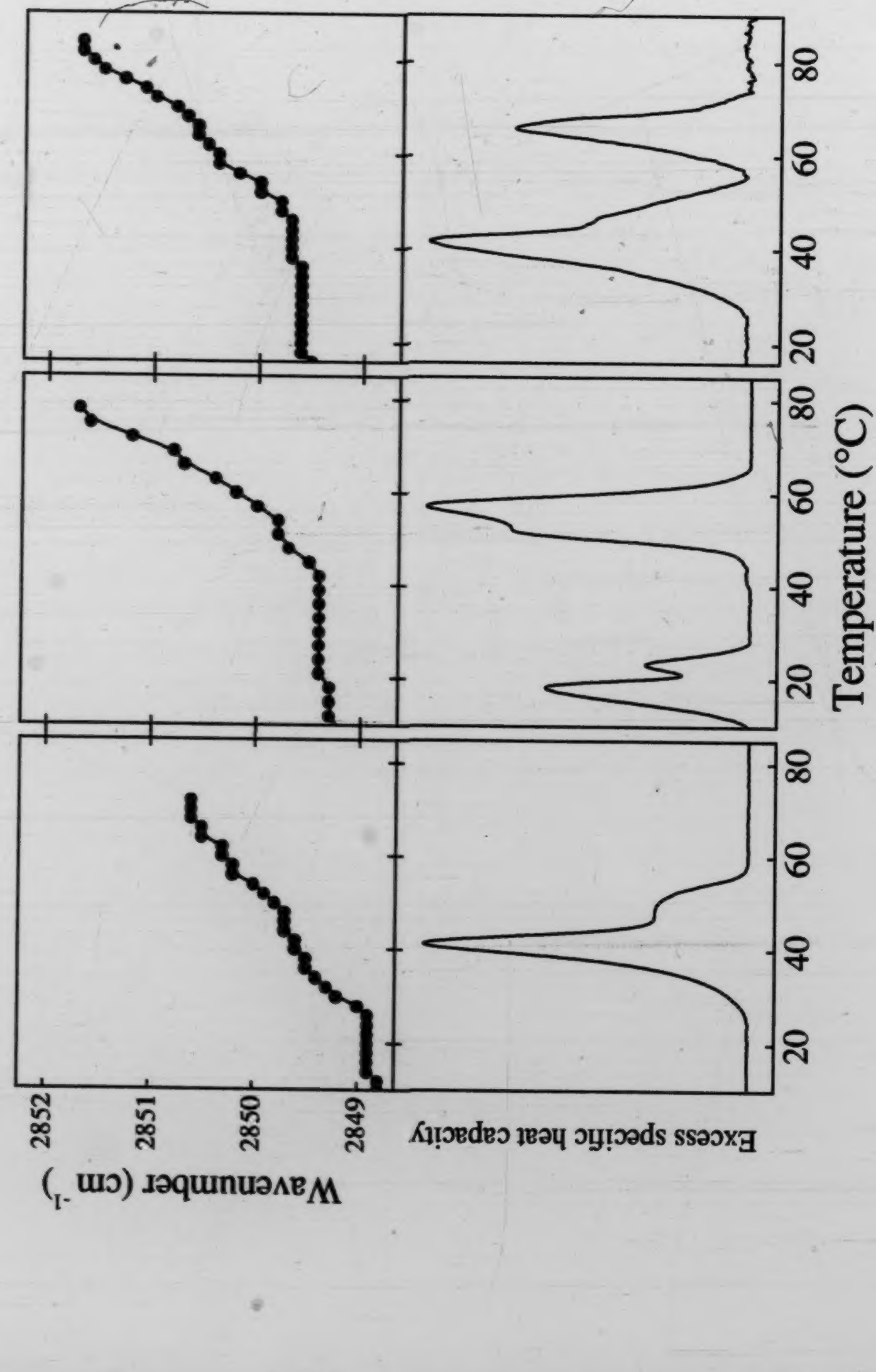
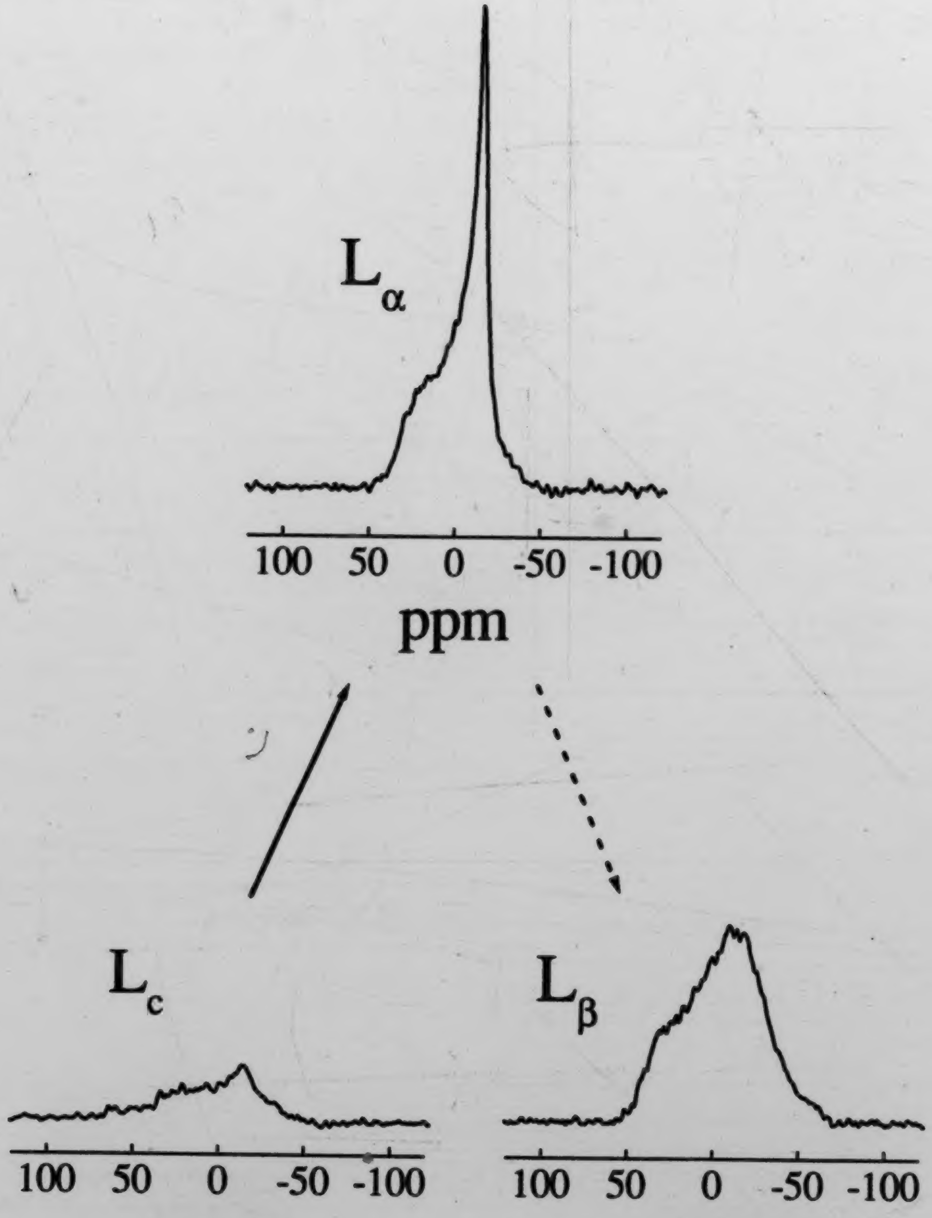

Wavenumber (cm $^{-1}$)
2852
2851
2850
2849
Excess specific heat capacity
20
40
60
80
20
40
60
80
20
40
60
80
Temperature (°C)

Figure VII-10. Representative ^{31}P-NMR spectra of pure PE bilayer L_α, L_β and L_c phases as labeled in the Figure. Arrows indicate L_β/L_α or L_c/L_α transitions. Illustrated spectra were obtained from fully hydrated DMPE bilayers with approximately 10 000 transients, and DPPE and DSPE bilayers are essentially identical. Arrows indicate phase changes with increasing (solid) and decreasing (dotted) temperature. Note that the residual signal observed in the L_c phase is from the small number of PE molecules in the L_β phase.



L_α
100 50 0 -50 -100
ppm
L_c
100 50 0 -50 -100
ppm
L_β
100 50 0 -50 -100
ppm

Figure VII-11. Representative ^{31}P-NMR spectra of DMPE bilayers containing >30 mol% cholesterol and DPPE bilayers containing >10 mol% cholesterol obtained upon heating and cooling as given by the directional arrows. See text for details on the variations in ^{31}P spectra lineshape and linewidth with changes in cholesterol concentration and PE chain length. Arrows indicate phase changes with increasing (solid) and decreasing (dotted) temperature. Signal for each spectra obtained with approximately 10 000 transients.

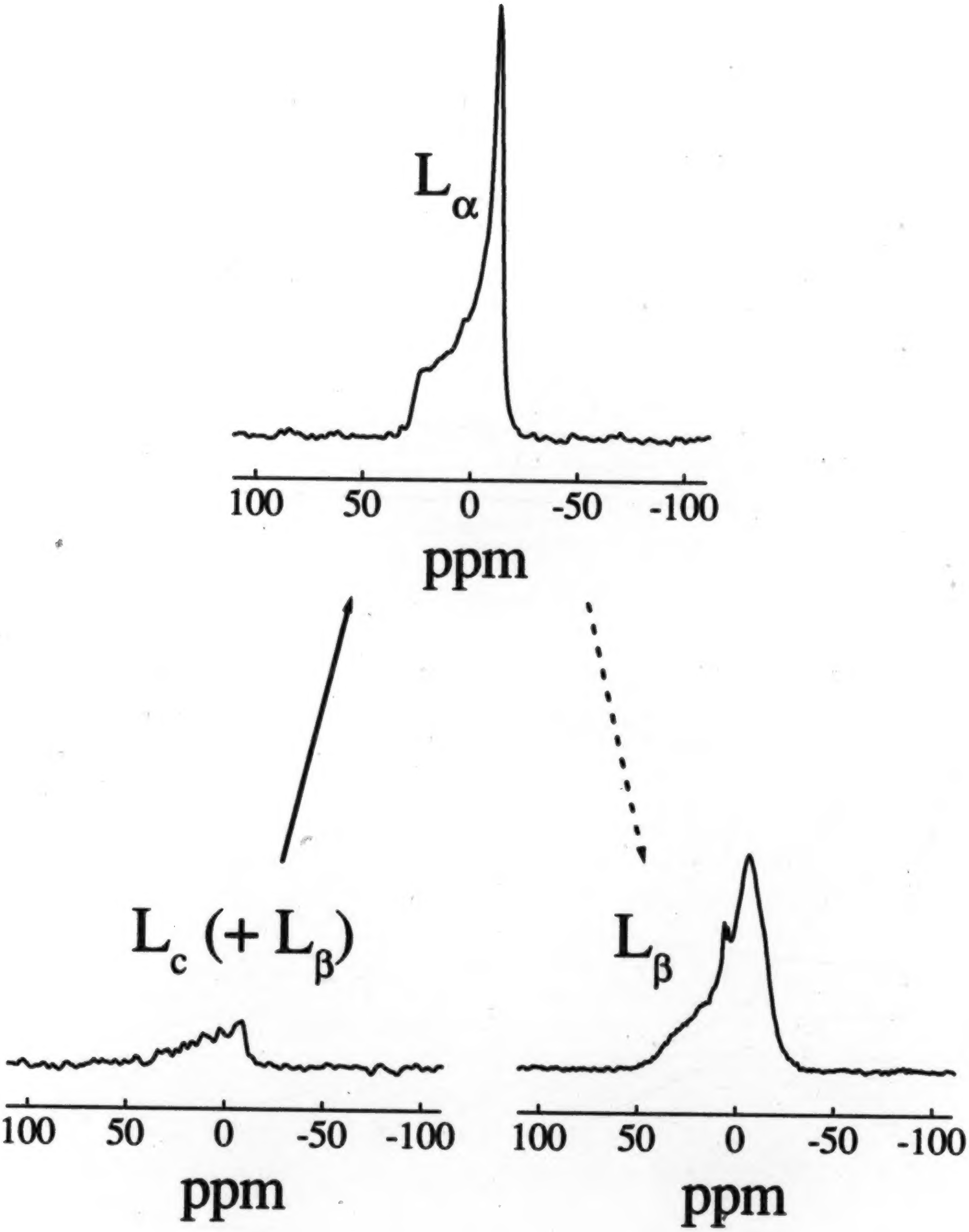
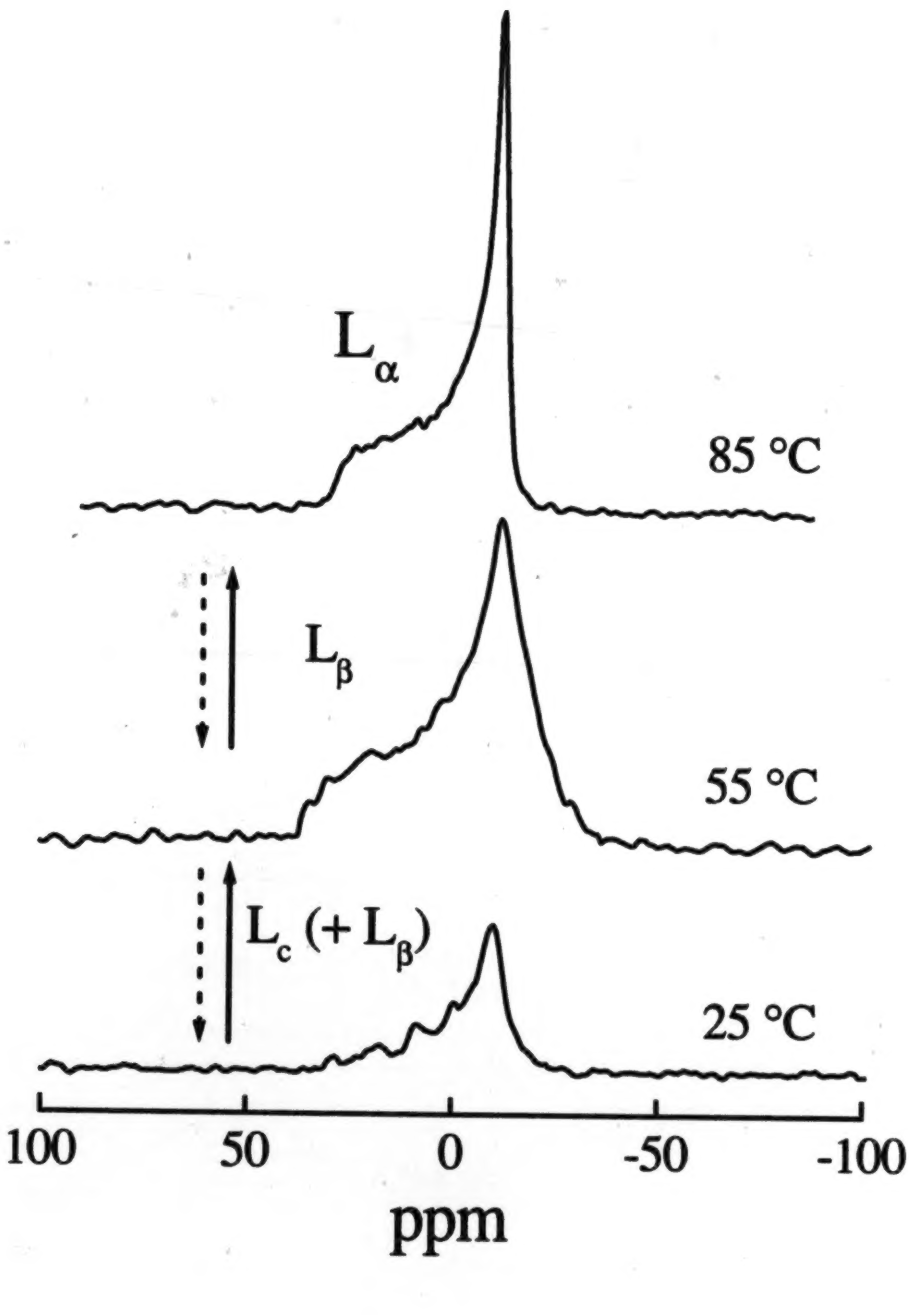

L_α
100
50
0
-50
-100
ppm
L_c (+ L_β)
100
50
0
-50
-100
ppm
L_β
100
50
0
-50
-100
ppm

Figure VII-12. Representative ^{31}P-NMR spectra of cholesterol/DSPE mixtures, inclusive. Arrows indicate phase changes with increasing (solid) or decreasing (dotted) temperature. See text for details on the variations in ^{31}P spectra lineshape and linewidth with changes in cholesterol concentration. Signal for each spectra obtained with approximately 10 000 transients.


L$_\alpha$
85 °C
L$_\beta$
55 °C
L$_c$ (+ L$_\beta$)
25 °C
100
50
0
-50
-100
ppm

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CHAPTER VIII. CALORIMETRIC AND SPECTROSCOPIC STUDIES OF THE EFFECTS OF CHOLESTEROL ON THE THERMOTROPIC PHASE BEHAVIOR AND ORGANIZATION OF A HOMOLOGOUS SERIES OF LINEAR SATURATED PHOSPHATIDYLSERINE BILAYERS[8]

INTRODUCTION

Cholesterol, or a closely related sterol, are major components of the plasma membranes of most eukaryotic cells and are also found in lower concentrations in some intracellular membranes (Nes and McKean, 1977; Yeagle, 1988). Thus a large number of studies of cholesterol/phospholipid bilayer model membranes, in particular cholesterol/DMPC and cholesterol/DPPC mixtures, have been carried out using a wide range of physicochemical techniques (Demel and de Kruijff, 1977; Yeagle, 1988; Vist and Davis, 1990; McMullen and McElhaney, 1996). Briefly, increasing levels of cholesterol abolish the cooperative gel to liquid-crystalline phase transition of PC bilayers while also decreasing the permeability of the bilayer and increasing its mechanical strength. Cholesterol-rich phospholipid domains are also characterized by phospholipid intra- and intermolecular motional rates which are similar to those of a fluid phospholipid bilayer, but with hydrocarbon chain orientational order and phospholipid area compressibility values which more closely resemble the gel phase of a pure phospholipid. The unique phase state of cholesterol-rich PC domains has been defined as the liquid-ordered or β-phase (Ipsen *et al.*, 1987; Vist and Davis, 1990). This liquid-ordered (β-) phase is postulated to be the relevant physical state of many biological membranes, such as the plasma membrane of eukaryotic cells, which contain cholesterol concentrations greater than 25 mol% of the total lipid (Vist and Davis, 1990; Thewalt and Bloom, 1992).

In addition to the PCs, most eukaryotic cell plasma membranes contain appreciable levels of the zwitterionic PE and anionic PS phospholipid species, each which may contain hydrocarbon chains ranging from 14 to 22 carbons in length, with various degrees of unsaturation (Ansell and Spanner, 1982). While, there are relatively few investigations of the effect of cholesterol on phospholipid bilayers of varying headgroup or hydrocarbon chain composition, it is clear that cholesterol-phospholipid interactions vary markedly with the structure of the phospholipid polar headgroup as well as the hydrocarbon chains (Demel *et al.*, 1977; van Dijck *et al.*, 1979; Blume, 1980; Blume and Griffin, 1982; Bach, 1984; Bach *et al.*, 1992; Wachtel and Bach, 1987; McMullen *et al.*, 1993; Borochov *et al.*, 1995). For example, DSC studies of cholesterol/PC mixtures revealed that the addition of cholesterol may either increase, have little effect on, or decrease the temperature of the PC chain-melting phase transition, depending on the length of the PC hydrocarbon chains and whether one or both of the hydrocarbon chains are unsaturated (Davis and Keough, 1983; Keough *et al.*, 1989; McMullen *et al.*, 1993; Kariel *et al.*, 1991; Vilchèze *et al.*, 1996). Moreover, the level of cholesterol required to abolish the chain-melting phase transition of the host PC bilayer may be less than (Davis and Keough, 1983; Keough *et al.*, 1989), equal to (McMullen *et al.*, 1993), or greater than (Kariel *et al.*, 1991; Vilchèze *et al.*, 1996) 50 mol% cholesterol, depending on the composition of the PC hydrocarbon chains. Similarly, cholesterol was reported either to markedly reduce (POPE and DEPE), moderately reduce (DPPE, EYPE), or have little effect on (DMPE), the temperature of the host PE bilayer chain-melting phase transition, while the level of cholesterol required to abolish the cooperative chain-melting phase transition of these same PE bilayers varies from as little as 30 mol% to levels exceeding 50 mol% cholesterol (van Dijck *et al.*, 1976; Blume, 1980; Epand and Bottega, 1987; Cheetham *et al.*, 1989). Furthermore, in contrast to cholesterol/PC and cholesterol/PE mixtures, Bach and coworkers reported that cholesterol had relatively limited effects on both the temperature and enthalpy of the chain-melting phase transitions of POPS, SOPS, DPPS and bovine brain PS, even at cholesterol levels of 50 mol% (Bach, 1984; Bach and Wachtel, 1987; Wachtel and Bach, 1989; Bach *et al.*, 1992). Interestingly, in both cholesterol/PS bilayers with both saturated and unsaturated acyl chains and cholesterol/PE mixtures with unsaturated acyl chains, but not in any cholesterol/PC mixture studied thus far, there is evidence for cholesterol-phospholipid solid phase separation at cholesterol levels of 20-30 mol% and higher (Epand and Bottega, 1987;

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Cheetham *et al.*, 1989; Bach, 1984; Bach and Wachtel, 1987; Bach and Wachtel, 1989; Bach *et al.*, 1992). However, there is no apparent relationship between the solubility of cholesterol and the resulting effect of cholesterol on the thermotropic phase behavior of the host PE or PS bilayer. In summary, the results of prior studies of cholesterol-phospholipid interactions are not consistent, nor do they provide a detailed picture of cholesterol-phospholipid interactions at the molecular level.

Prior studies examining the effect of phospholipid polar headgroup structure on cholesterol-phospholipid interactions often utilize phospholipids of varying hydrocarbon chain length and saturation. However, we demonstrated recently in both saturated and unsaturated PC and PE bilayers that variations in cholesterol-phospholipid hydrophobic mismatch can markedly alter the miscibility of cholesterol in the host bilayer and the subsequent effect of cholesterol on the chain-melting phase transition of the host bilayer (McMullen *et al.*, 1993, 1996a,b; Vilchèze *et al.*, 1996). Similarly, the interactions of SPM and GalCer with cholesterol were also found to depend on the hydrocarbon chain composition and phase state of the host phosphosphingolipid or glycosphingolipid bilayer (Ali *et al.*, 1994; Smaby *et al.*, 1995). Thus, to properly assess the contribution of the phospholipid polar headgroups to cholesterol-phospholipid interactions, we have examined the effect of cholesterol on the thermotropic phase behavior and organization of DMPS, DPPS and DSPS bilayers using HS-DSC, FTIR and ^{31}P-NMR spectroscopy. This study is designed to complement similar comprehensive studies of the effect of cholesterol on the other major eukaryotic phospholipids, the PCs (McMullen *et al.*, 1993) and PEs (McMullen *et al.*, 1996). We reveal that the effect of cholesterol on the enthalpy, cooperativity and temperature of the PS main chain melting transition depends whether monitored in the heating or cooling mode as well as on the hydrocarbon chain length of the host PS bilayers. In addition, the incorporation of cholesterol in PS bilayers appears to facilitate the lateral phase separation of cholesterol and PS, as well as the formation of highly ordered, crystalline PS phospholipid domains. Thus, we find that the interaction of cholesterol with PS bilayers differs significantly from that documented for corresponding cholesterol/PC mixtures (McMullen *et al.*, 1993). Moreover, in conjunction with McMullen *et al.* (1993, 1996), we demonstrate how variations in the composition of the phospholipid polar headgroup and hydrocarbon chains contribute to differences in the lateral distribution of cholesterol in the host phospholipid bilayer.

MATERIALS AND METHODS

The PSs used in these experiments were purchased from Avanti Polar Lipids (Alabaster, AB) and checked for purity by TLC using chloroform/methanol/glacial acetic acid/water (60:40:10:4, by volume) as the developing solvent followed by spraying with 2% K_2CrO_4 in 60% sulfuric acid and charring. The cholesterol was also purchased from Avanti Polar Lipids and recrystallized from ethanol before use. For cholesterol/DMPS and cholesterol/DPPS mixtures, stock solutions of PS and cholesterol in chloroform/methanol (2:1, vol/vol) were used to prepare the cholesterol/PS mixtures. After addition of the appropriate amounts of PS and cholesterol from the respective stock solutions, the samples were vortexed and heated to approximately 40 to 50 °C under N_2 to remove the solvent and then dried under vacuum for at least 18 hours. Due to the relatively low solubility of DSPS in mixtures of chloroform and methanol, we prepared cholesterol/DSPS mixtures by weighing out the appropriate amounts of DSPS and cholesterol, dissolved the powders in benzene, gently heated, and then lyophilized. The samples were dried under vacuum for at least 18 hours to ensure that all traces of the benzene was removed.

For the HS-DSC experiments the dried cholesterol/PS mixtures were dispersed in a buffer containing 50 mM Tris, 100 mM NaCl, 10 mM EDTA (pH 7.4) and heated to approximately 10-20°C above the phase transition of the mixture (for at least 30 minutes) with repeated vortexing to give a multilamellar suspension. When we examined the effect of variations in buffers (Tris and phosphate) and ionic strength (NaCl 0.100 to 0.400 M) on the thermotropic phase behavior of cholesterol/PS mixtures, we observed only small shifts in the transition temperature and no changes in the qualitative behavior of the sample. For all of the HS-DSC samples containing cholesterol, the mixtures were hydrated and suspended as detailed, then stored for 1 day at 2 °C before the HS-DSC experiment. The HS-DSC thermograms for the PS/cholesterol suspensions were recorded with a Hart

7701 high-sensitivity differential scanning calorimeter (Provo, UT). The scan rates used were 10 °C/hr unless otherwise noted. In addition, the amount of PS used for DSC was progressively increased from 0.5 mg for pure PS bilayers to 20 mg for PS samples containing 45 or 50 mol% cholesterol. We have shown previously that this protocol is required to accurately monitor the broad, low-enthalpy phase transitions observed at higher cholesterol concentrations (McMullen *et al.*, 1993). The Hart calorimeter was calibrated using solid standards from Hart Scientific as well as aqueous lipid samples synthetically prepared and purified within this laboratory using methods previously shown to provide highly pure samples (Lewis *et al.*, 1985). Sample runs were repeated at least three times to ensure reproducibility. For all mixtures phospholipid and cholesterol degradation was monitored by TLC and no degradation products were observed. Moreover, sequential HS-DSC runs were completely reproducible, supporting the absence of chemical degradation in our samples after HS-DSC or spectroscopic analysis. The analysis and the decomposition of the HS-DSC endotherms was done using Microcal's Origin (Northhampton, MA) and DA-2 software.

For FTIR spectroscopic analysis, the cholesterol/PS dispersions were prepared as described above, including 1 day preincubation, for the HS-DSC experiments except these samples were suspended in D_2O buffered with 100 mM sodium phosphate, 100mM NaCl and 10 mM EDTA (pH 7.4). The samples were placed between CaF_2 windows containing a 25-um spacer and mounted in a cell holder attached to a computer-controlled circulating water bath. FTIR spectra were recorded with a Digilab (Cambridge, MA) FTS-40 Fourier transform infrared spectrometer. Temperature-induced shifts in the absorption maxima and bandwidth of the CH_2 symmetric and asymmetric stretching bands near 2850 cm^{-1} and 2920 cm^{-1}, respectively, were used to monitor changes in the conformational state of the hydrocarbon chains of the PS molecules (Lewis and McElhaney, 1993; Mendelsohn and Mantsch, 1986; Snyder, 1967; Mantsch and McElhaney, 1991). Alterations in the frequency of the CH_2 scissoring band near 1468 cm^{-1} indicated changes in the solid-state PS hydrocarbon chain packing (Lewis and McElhaney, 1993; Snyder, 1961, 1979). In addition, we monitored the absorption of the phospholipid carbonyl stretching band centered at 1735-1745 cm^{-1}, which assisted in the identification of PS bilayers exhibiting relatively dehydrated interfaces typical of lamellar gel or crystalline phases (Lewis and McElhaney, 1993). The data was processed using computer programs developed by the National Research Council of Canada.

RESULTS

Effect of cholesterol on the thermotropic phase behavior and organization of DMPS, DPPS and DSPS bilayers: Calorimetric studies.

The effect of cholesterol on the thermotropic phase behavior of PS bilayers clearly depends on the chain length of the PS molecule as well as whether or not the thermotropic behavior was monitored on heating or cooling. In the absence of cholesterol and in both the heating and cooling modes, DMPS bilayers exhibit the highly cooperative and energetic L_β to L_α chain melting transition at 39.5 °C, with an enthalpy value of approximately 7.8 kcal/mol. Shown in Figure VIII-1 are the heating (left panel) and cooling (right panel) HS-DSC scans, at 10 °C/hr, of DMPS bilayers containing progressively increasing levels of cholesterol. When monitored upon heating, the addition of cholesterol has little affect on the L_β/L_α transition temperature (see Figure VIII-2), but markedly reduces the enthalpy (see Figure VIII-3) and cooperativity (Figure VIII-1) of the DMPS chain-melting phase transition. At cholesterol levels approaching 50 mol%, the L_β/L_α transition is virtually abolished. However, in addition to the chain-melting phase transition, we also noted the appearance of a lower temperature, lower enthalpy transition at cholesterol levels exceeding 5 mol% cholesterol in DMPS/cholesterol mixtures (see Figure VIII-1). The temperature (Figure VIII-2) and enthalpy (Figure VIII-3) of this transition corresponds well with the L_c/L_β transition observed in DMPS bilayers which have been annealed for long periods of time at low temperature (results not shown) (Lewis and McElhaney, 1993; Hubner *et al.*, 1994). Thus the addition of cholesterol actually facilitates the formation of the L_c phase in DMPS bilayers and this effect is maximal at 10 mol% cholesterol. Therefore, at low temperatures both the cholesterol-rich PS L_β and cholesterol-poor PS L_c phases coexist in cholesterol/DMPS bilayers. Interestingly, when we progressively increased the scan rate of the HS-DSC scans in the heating mode from 10 to 60 °C, the enthalpy of the low temperature L_c/L_β transition progressively decreased, and the L_c/L_β transition is no longer observed at scan rates of 60 °C/hr in any of the cholesterol/DMPS mixtures (results not shown). In contrast, when the thermotropic phase behavior of cholesterol/DMPS mixtures is monitored on cooling, cholesterol progressively decreases the enthalpy (see Figure VIII-3) and cooperativity (Figure VIII-1) of the DMPS chain-melting phase transition with only a slight decrease in transition temperature (see Figure VIII-2), and by 50 mol% cholesterol this transition is completely abolished. Unlike the heating scans, the thermotropic behavior of cholesterol/DMPS mixtures does not vary with scan rate on cooling, nor were any additional endothems observed.

Shown in Figure VIII-4 are the representative HS-DSC heating (left panel) and cooling (right panel) scans for DPPS bilayers with progressively increasing amounts of cholesterol. In the absence of cholesterol and in the heating mode, DPPS bilayers exhibit an L_c/L_β phase transition at approximately 34 °C (1.3 kcal/mol) and the L_β/L_α chain-melting phase transition at 50 °C with an enthalpy value of 9.6 kcal/mol (see also Hubner *et al.*, 1994). On cooling, only the L_β/L_α chain-melting phase transition is observed. Upon the addition of progressively increasing levels of cholesterol to DPPS bilayers (in the heating mode), the temperature (see Figure VIII-5) of the L_β/L_α transition decreases only slightly and the cooperativity (Figure VIII-4) and enthalpy (Figure VIII-6) of the DPPS chain-melting phase transition are moderately reduced. Similarly, the temperature (Figure VIII-5) and cooperativity (Figure VIII-4) of the L_c/L_β transition does not vary significantly with increasing cholesterol incorporation and the enthalpy (Figure VIII-6) of this transition decreases only moderately. However, we did note that when we progressively increased the scan rate in the heating mode, the enthalpy of the low temperature L_c/L_β phase transition of cholesterol/DPPS mixtures decreases markedly (results not shown). When monitored in the cooling mode, increasing levels of cholesterol progressively decrease the chain-melting phase transition enthalpy (see Figure VIII-6), temperature (Figure VIII-5) and cooperativity (Figure VIII-4) of the DPPS chain-melting transition, but the transition is still apparent at 50 mol% cholesterol (Figure VIII-4). We also found additional low enthalpy transitions of unknown origin at 25-27 °C in DPPS bilayers containing 35 and 50 mol% cholesterol. The thermotropic behavior of the cholesterol/DPPS mixtures when observed in the cooling mode is not affected by increases in scan rate.

Shown in Figure VIII-7 are the representative endotherms for the chain-melting phase transition of cholesterol/DSPS bilayers as a function of increasing cholesterol concentration upon heating (left panel) and cooling (right panel). Upon heating, in the absence of cholesterol, DSPS bilayers exhibit an L_c/L_β transition at approximately 45 °C (1.6 kcal/mol) and an L_β/L_α chain-melting transition at 62.5 °C (12 kcal/mol). On cooling only the L_β/L_α chain-melting transition is observed. When monitored on heating, the incorporation of cholesterol in DSPS bilayers has little impact on the temperature (see Figure VIII-8) but progressively decreases the cooperativity (Figure VIII-7) and enthalpy (Figure VIII-9) of the chain-melting phase transition of DSPS bilayers, and by 50 mol% cholesterol this transition is virtually abolished. Conversely, the addition of cholesterol decreases the temperature (Figure VIII-8) but increases the enthalpy (Figure VIII-9) of the lower temperature L_c/L_β transition of cholesterol/DSPS mixtures. At 20 mol% cholesterol, an additional low temperature endotherm appears, centered at 35 °C, which increases markedly in size (Figure VIII-7) with increasing cholesterol levels. At cholesterol levels approaching 50 mol%, the transition at 35 °C dominates (Figures VIII-7 and VIII-9), and the chain-melting phase transition is only barely apparent on the heating scans. As in cholesterol/DMPS and cholesterol/DPPS mixtures, increasing the scan rate in the heating mode progressively decreased the enthalpy of both the low-temperature and L_c/L_β transitions. When monitored in the cooling mode, the temperature (Figure VIII-8) of the chain-melting phase transition of cholesterol/DSPS mixtures does not vary significantly with progressively increasing cholesterol levels, but the cooperativity (Figure VIII-7) and enthalpy (Figure VIII-9) are markedly reduced and by approximately 50 mol% cholesterol, the transition is virtually abolished. As in both cholesterol/DMPS and cholesterol/DPPS mixtures, increasing the scan rate had little affect on the thermotropic behavior of cholesterol/DSPS mixtures when monitored on cooling.

Effect of cholesterol on the thermotropic phase behavior and organization of DMPS, DPPS and DSPS bilayers: FTIR spectroscopic studies.

We performed FTIR spectroscopic analyses of cholesterol/PS mixtures to determine the effect of cholesterol on the phase state and conformational disorder of the PS/cholesterol bilayers as a function of temperature and to correlate this information with the observed calorimetric transitions. Both chain-melting (L_β/L_α) and gel state polymorphic (L_c/L_β) phase transitions of phospholipid bilayers are accompanied by distinct changes in the CH_2 stretching, CH_2 scissoring and C=O stretching absorption bands (Hubner *et al.*, 1994). It is important to note that the thermal history of the cholesterol/PS samples for FTIR and HS-DSC analysis were virtually identical so as to ensure a reasonable comparison of the results from these two techniques.

Shown in Figure VIII-10 are representative FTIR spectra of the CH_2 stretching (left panel), the C=O stretching (middle panel), and CH_2 scissoring (right panel) absorption regions of aqueous dispersions of pure PS bilayers for the L_c1, L_c2, L_β and L_α phases. Note that the changes in the phase state and organization of the bilayers result in distinctive contours for each monitored band. PS bilayers in the L_c (type 1 or 2) or L_β phases exhibit CH_2 stretching symmetric and asymmetric band absorption maxima at 2848 and 2920 cm^{-1}, respectively (Figure VIII-10, left panel). When the PS bilayer converts to the L_α phase, the maxima shift up approximately 3 cm^{-1}. The C=O stretching band of the L_c1 phase of PS bilayers is a composite of three bands at approximately 1738, 1718 and 1708 cm^{-1}, while two distinct individual bands at approximately 1740 and 1718 cm^{-1} characterize the L_c2 phase of PS bilayers (Figure VIII-10, middle panel). The PS L_β and L_α phases exhibit C=O stretching bands with underlying maxima at approximately 1742 and 1724 cm^{-1}, the primary difference between the two phases being the considerably broader width of the C=O stretching band of the L_α phase. Lastly, in the CH_2 scissoring band (Figure VIII-10, right panel), the L_c1 and L_c2 phases exhibit band splitting with maxima at approximately 1473 and 1464 cm^{-1}, while the L_β phase exhibits a single, relatively sharp band at 1468 cm^{-1}. When the L_c1, L_c2 or L_β phases convert to the L_α phase, the band absorption maxima broadens dramatically and shifts down to approximately 1468 cm^{-1} and a band representing CH_3 symmetric scissoring at 1453-1456 cm^{-1} becomes prominent. Note that the formation of the L_c type 1 or 2 phases in pure PS bilayers typically required extended periods of low temperature incubation (> 1 week) to ensure growth of PS crystalline phases.

In the absence of cholesterol, the FTIR spectra of DMPS bilayers annealed at low temperatures for a prolonged time period (> 1 week) are consistent with the L_c1 phase, as shown in Figure VIII-10. With increasing temperature, the L_c1 phase converts to the L_β phase at approximately 26 °C which in turn converts to the L_α phase at approximately 40 °C (see Figure VIII-10). DMPS mixtures not subject to prolonged low temperature incubation, or monitored in the cooling mode, exhibit only the L_β/L_α phase transition at approximately 40 °C. Upon the incorporation of 5 to 45 mol% cholesterol in DMPS bilayers (not subject to prolonged low temperature incubation), we observe spectra consistent with both the lower temperature L_c1/L_β and higher temperature L_β/L_α phase transitions, as shown in Figure VIII-11. The temperatures of the L_c1/L_β and L_β/L_α conversions are fully consistent with that observed in the corresponding HS-DSC scans. The splitting of the CH_2 scissoring band observed in the L_c1 phase of cholesterol/DMPS mixtures is particularly significant because it suggests that the PS hydrocarbon chains are packed in an orthorhombic perpendicular subcell (Synder 1961, 1979). Since the correlation field splitting can only occur when there are large domains of orthorhombic packed all-*trans* polymethylene chains, we conclude that relatively large domains of virtually pure PS molecules exist even in the presence of high (45 mol%) cholesterol concentrations. It is also important to note that the time required for DMPS bilayers to form the L_c1 phase (> 1 week) is considerably longer than that observed for cholesterol/DMPS mixtures (< 24 hrs) and cholesterol thus appears to facilitate the formation of highly ordered crystalline phases in at low temperature. In addition, the C=O stretching and CH_2 scissoring absorption band spectra for low temperature cholesterol/DMPS mixtures likely represent a mixture of cholesterol-rich L_β and cholesterol-poor L_c1 phospholipid domains, which would explain the small differences between these absorption spectra and those shown for the pure PSs in Figure VIII-10. In contrast to the heating scans, in the cooling mode all cholesterol/DMPS mixtures (5 to 45 mol%) exhibit an L_α/L_β transition, as indicated by representative spectra in Figure VIII-12. The only difference between the various cholesterol/DMPS mixtures was the temperature range of the phase conversion, which progressively increased with increasing cholesterol levels, consistent with the decreasing cooperativity of the L_α/L_β phase transition as observed by HS-DSC cooling scans (Figure VIII-1).

In the absence of cholesterol, the FTIR spectra of DPPS bilayers annealed at low temperatures for a prolonged time period (> 1 week) are consistent with the L_c2 phase, as shown in Figure VIII-10. With increasing temperature, the L_c2 phase converts to the L_β phase at approximately 34 °C, which in turn converts to the L_α phase at approximately 52 °C (see Figure VIII-10). DPPS mixtures not subject to prolonged low temperature incubation, or monitored in the cooling mode, exhibit only the L_β/L_α phase transition at approximately 52 °C. FTIR analysis revealed that cholesterol/DPPS mixtures (not subject to prolonged low temperature incubation) at all cholesterol levels examined (5 to 50 mol%), undergo both L_c2/L_β and L_β/L_α transitions (Figure VIII-13) at temperatures fully consistent with HS-DSC. The small differences observed in either the DPPS C=O stretching or CH_2 scissoring absorption bands at different cholesterol concentrations relative to that observed for pure PS bilayers are likely due to the presence of both cholesterol-rich L_β and cholesterol-poor L_c2 PS domains in the cholesterol/DPPS mixture at low temperatures. When the C=O stretching and CH_2 scissoring bands (Figure VIII-12) are monitored on cooling for cholesterol/DPPS mixtures, we observed only L_α/L_β phase conversion, consistent with the temperature range of the exotherm shown by HS-DSC in corresponding cholesterol/DPPS mixtures. No further information was obtained on the nature of the additional low temperature transitions that were observed on cooling by HS-

DSC at approximately 23 °C in cholesterol/DPPS mixtures with 35 and 50 mol% cholesterol (see Figure VIII-4).

In the absence of cholesterol, the FTIR spectra of DSPS bilayers annealed at low temperatures for a prolonged time period (> 1 week) are consistent with the L_c2 phase, as shown in Figure VIII-10. With increasing temperature, the L_c2 phase converts to the L_β phase at approximately 46 °C, which in turn converts, with further increases in temperature, to the L_α phase at approximately 64 °C (see Figure VIII-10). DSPS mixtures not subject to prolonged low temperature incubation, or monitored in the cooling mode, exhibit only the L_β/L_α phase transition at approximately 64 °C. In cholesterol/DSPS mixtures (not subject to prolonged low temperature incubation) with cholesterol levels from 5 to 20 mol%, we clearly observe the formation of the L_c2 phase (see Figure VIII-13), which then converts to the L_β phase followed by the L_α phase with increased temperature. These results are fully consistent with our HS-DSC results. However, under the same conditions (identical sample preincubation time and FTIR scanning protocol), the L_c2 phase becomes progressively less prominent as the level of cholesterol increases towards 50 mol%. Representative spectra for DSPS bilayers containing 35 mol% cholesterol are shown in Figure VIII-14. At low temperatures it is clear that the L_β phase predominates and increasing temperature does not alter the C=O stretching and CH_2 scissoring bands until the chain-melting transition at approximately 62 °C, which is also broad and difficult to discern, consistent with our HS-DSC results. Since we do not observe DSC endotherms characteristic of the anhydrous cholesterol polymorphic transition at approximately 38 °C, nor the cholesterol monohydrate/anhydrous phase transition at 86 °C (Loomis *et al.*, 1979), the present spectroscopic and calorimetric evidence suggest that the low-temperature endotherm in cholesterol/DSPS mixtures, in the heating mode, represents a cooperative lateral reorganization of cholesterol in the DSPS bilayer, similar to that documented for androstenol/PC and cholesterol/PE mixtures by this laboratory (McMullen *et al.*, 1994; McMullen *et al.*, 1996). Conversely, in the cooling mode, the C=O stretching and CH2 scissoring bands of cholesterol/DSPS mixtures are consistent with a single L_α/L_β phase transition (see Figure VIII-12). Moreover, as the cholesterol level approaches 50 mol% in DSPS bilayers, an L_α/L_β phase transition is no longer discernible by FTIR on cooling.

DISCUSSION

It is clear from this study, and a corresponding study of cholesterol/PC mixtures (McMullen *et al.*, 1993), that the interaction of cholesterol varies markedly with the structure of both the phospholipid polar headgroup and hydrocarbon chains. In cholesterol/PC mixtures of varying hydrophobic chain length, which exhibit relatively weak attractive PC inter-headgroup hydrogen bonding and electrostatic interactions, cholesterol appears to be fully and equally miscible in the gel and liquid-crystalline states, with an effective stoichiometry of 3.5 PC molecules per cholesterol molecule (Engleman and Rothman, 1972). Thus, on both heating and cooling scans, the effect of increasing levels of cholesterol is to progressively decrease the cooperativity and enthalpy of the PC chain-melting enthalpy, such that by 50 mol% the transition is abolished. Conversely, PS bilayers exhibit extensive inter-headgroup hydrogen bonding and electrostatic interactions, especially at low temperatures and in the crystalline phase (Boggs, 1987). As a consequence of the higher order and tighter packing of PS phospholipids relative to PCs (Tutenhagen *et al.*, 1994), the miscibility of cholesterol in the host PS bilayer is considerably reduced from that observed in corresponding PC bilayers, especially at low temperatures and higher cholesterol concentrations. Thus, by favoring phospholipid-phospholipid contacts, the lateral exclusion of cholesterol is favored at low temperatures which allows for the formation of cholesterol-poor PS domains which can then form highly ordered crystalline phases (see Lewis and McElhaney, 1993; Hubner *et al.*, 1994). Furthermore, increasing PS hydrocarbon chain length also appears to accelerate the lateral phase separation of cholesterol-PS mixtures at low temperatures, presumably due to the increased attractive van der Waals interactions between neighboring PS hydrocarbon chains. The sequestering of cholesterol at the domain boundaries of crystalline PS domains also appears to permit coexisting cholesterol-rich L_β PS and cholesterol-poor L_c PS domains in cholesterol/PS mixtures, even at cholesterol levels of 50 mol%. The identification of cholesterol-poor crystalline PS domains at low temperature in cholesterol/PS mixtures, as well as their coexistence with gel state cholesterol-rich phospholipid domains, is in marked contrast to the disordering effect of cholesterol on the hydrocarbon chains of PC bilayers in the gel state. Moreover, due to the decreased effective stoichiometry of cholesterol-PS interactions, the magnitude of the effect of cholesterol on the enthalpy, cooperativity and temperature of the gel/gel and chain-melting phase transitions of PS bilayers on heating is also markedly attenuated relative to corresponding cholesterol/PC mixtures. As the temperature of the cholesterol/PS mixture increases and eventually surpasses the temperature of the L_c/L_β and L_β/L_α transitions, the increased hydrocarbon chain disorder and intermolecular motion reduces attractive intermolecular interfacial electrostatic and hydrogen bonding as well as non-polar van der Waals interactions. As a result the intercalation of cholesterol between PS molecules becomes more favorable and the miscibility of cholesterol in the host PS bilayer increases markedly. The resulting increase in the number of effective cholesterol-PS contacts in the liquid-crystalline phase results in the more pronounced effect of cholesterol on the cooperativity and enthalpy of the host phospholipid bilayer chain melting transition when monitored in the cooling mode. Thus, cholesterol-PS contacts are maximized and more consistent with the steric model of cholesterol-phospholipid interactions at 3.5 phospholipids per cholesterol, which is observed in the corresponding cholesterol/PC mixtures (Engleman and Rothman, 1973; McMullen *et al.*, 1993). Interestingly, the results obtained with cholesterol/PS mixtures are qualitatively similar to those observed with corresponding cholesterol/PE mixtures (McMullen *et al.*, 1996) and PE molecules are also characterized by strong attractive hydrogen-bonding and electrostatic interactions. We therefore conclude that the miscibility of cholesterol with any particular phospholipid of a given hydrocarbon chain length, will be inversely related to the propensity for hydrogen bonding and electrostatic interactions of the phospholipid headgroups.

The temperature- and time-dependent effect of cholesterol on the phase behavior and organization of PE and PS bilayers also presents an interesting series of problems from an experimental perspective. For example, the relatively long times required to obtain data using NMR spectroscopy or X-ray crystallography will allow for extensive cholesterol-phospholipid lateral phase separation at low temperatures and the formation of the crystalline phases in both PE and PS bilayers during the timescale of the experiment, especially at high cholesterol levels. Conversely, most prior calorimetric experiments typically undergo repeated heating and cooling experiments with scan rates of at least 1 to as fast as 5 °C/min, thus the metastable crystalline state does not convert, or only partially converts, to the crystalline phase. Differences in sample handling and analysis may account for the discrepancy between the results of the present study and those of Bach and Wachtel (1989), which reported the presence of solid phase cholesterol in cholesterol/DPPS mixtures. We did not find any evidence for solid phase cholesterol in our cholesterol/PS mixtures, however, we did note that the transition temperatures of the various L_c/L_β transitions in cholesterol/DPPS mixtures are generally within 10 °C of the anhydrous polymorphic transition of cholesterol. Thus, in the absence of FTIR data, Bach and Wachtel (1989) may have interpreted the gel/gel transition of cholesterol/DPPS mixtures as the polymorphic transition of anhydrous cholesterol (Bach and Wachtel, 1989). In addition, the use of different techniques also lead to considerable differences in the observed effect of cholesterol on the thermotropic phase behavior and organization of PE bilayers (Blume, 1980; Blume and Griffin, 1982; McMullen *et al.*, 1996). Thus, the present protocol, which uses both spectroscopic and calorimetric techniques in the heating and cooling modes, is well suited to examine the dynamic phase behavior and organization of cholesterol/PE and cholesterol/PS mixtures which exhibit a marked dependence on temperature and thermal history.

Overall, our model for the differential affinity of cholesterol for different phospholipid bilayers is based on the degree of cholesterol-phospholipid hydrophobic mismatch and the ability of the phospholipid headgroups to participate in inter-headgroup hydrogen bonding. Thus, the miscibility of cholesterol in different host phospholipid bilayers decreases in the order PC>PS>PE, and with increasing cholesterol-phospholipid hydrophobic mismatch (McMullen *et al.*, 1993, 1996). In addition we have demonstrated that the stoichiometry of cholesterol-PE and cholesterol-PS interactions will vary markedly with temperature and the level of cholesterol, in contrast to the consistent stoichiometry of cholesterol-PC interactions (McMullen *et al.*, 1993; Chong, 1994; Tang *et al.*, 1995). Interestingly, the same appears to be true for the miscibility of transmembrane peptides with PC and PE (Zhang *et al.*, 1992; 1995a,b) and PS (unpublished observations from this laboratory) bilayers. Thus, the differential affinity of cholesterol, or transmembrane peptides, for different phospholipids may form the basis for domains of phospholipids selectively enriched or depleted in cholesterol and/or membrane proteins. Selective enrichment of cholesterol in particular phospholipid domains, based on exclusion from PS and PE domains or enrichment in PC domains, has been postulated to regulate steady state levels of cholesterol in different membranes or phospholipid vesicles (Yeagle and Young, 1986; van Meer, 1993; Welti and Glaser, 1994; Schroeder *et al.*, 1995). Differences in the composition of cholesterol-rich and cholesterol-poor phospholipid domains in cell membranes may also allow for the selective association of particular membrane proteins to particular regions of the membrane. For example the membrane protein caveolin, which is postulated to be involved in cholesterol transport in eukaryotic cells, selectively interacts with cholesterol-rich PC domains, (Murata *et al.*, 1995; Lange and Steck, 1996). Moreover our results also support a mechanism for the sorting of cholesterol and membrane proteins between different membranes as a consequence of the differences in the physical properties of cholesterol-rich and cholesterol-poor phospholipid domains in eukaryotic cells (Bretscher and Munro, 1993; van Meer, 1993; Welti and Glaser, 1994; Schroeder *et al.*, 1995). The coexistence of cholesterol-rich and cholesterol-poor phospholipid domains may also be important in membrane budding or endocytosis, as these processes have been shown to be facilitated by domain boundaries (Devaux, 1991; Dobereiner *et al.*, 1993). In summary, we have shown that the dynamic and heterogeneous organization of cholesterol-containing phospholipid bilayers is a consequence of the physical chemistry of phospholipid-phospholipid and cholesterol-phospholipid interactions. Our results support models for distinct cholesterol domains in biological membranes based on differences in the affinity of cholesterol for different phospholipids (see Thompson *et al.*, 1992; Glaser, 1993; Welti and Glaser, 1994; Vaz and Alameida, 1993; Schroeder *et al.*, 1995; Kinnunen *et al.*, 1996). This is in contrast to the postulated homogeneous and temperature-independent distribution of cholesterol in eukaryotic cell plasma membranes based on studies of cholesterol/PC mixtures (Ipsen *et al.*, 1987; Vist and Davis, 1990; Thewalt and Bloom, 1992).

It is clear that studies of cholesterol/DMPC and cholesterol/DPPC mixtures, regardless of their scope and depth, are not sufficient to describe the function of cholesterol in biological membranes. The complex composition of biological membranes, and the correspondingly large number of different cholesterol-phospholipid interactions, requires studies of cholesterol-phospholipid interactions require a much broader base of phospholipid structures. Thus, this laboratory will continue to examine the effect of cholesterol on the thermotropic phase behavior of phospholipid bilayers varied systematically with respect to the composition of the polar headgroup and hydrocarbon chains.

Figure VIII-1: Representative heating (left panel) and cooling (right panel) thermograms of DMPS bilayers containing progressively increasing levels of cholesterol as indicated on the Figure. HS-DSC scans of DMPS bilayers containing 15 and 25 mol% cholesterol were also performed but are not shown. All endotherms shown were scanned at 10 °C/hr and are normalized for sample mass.

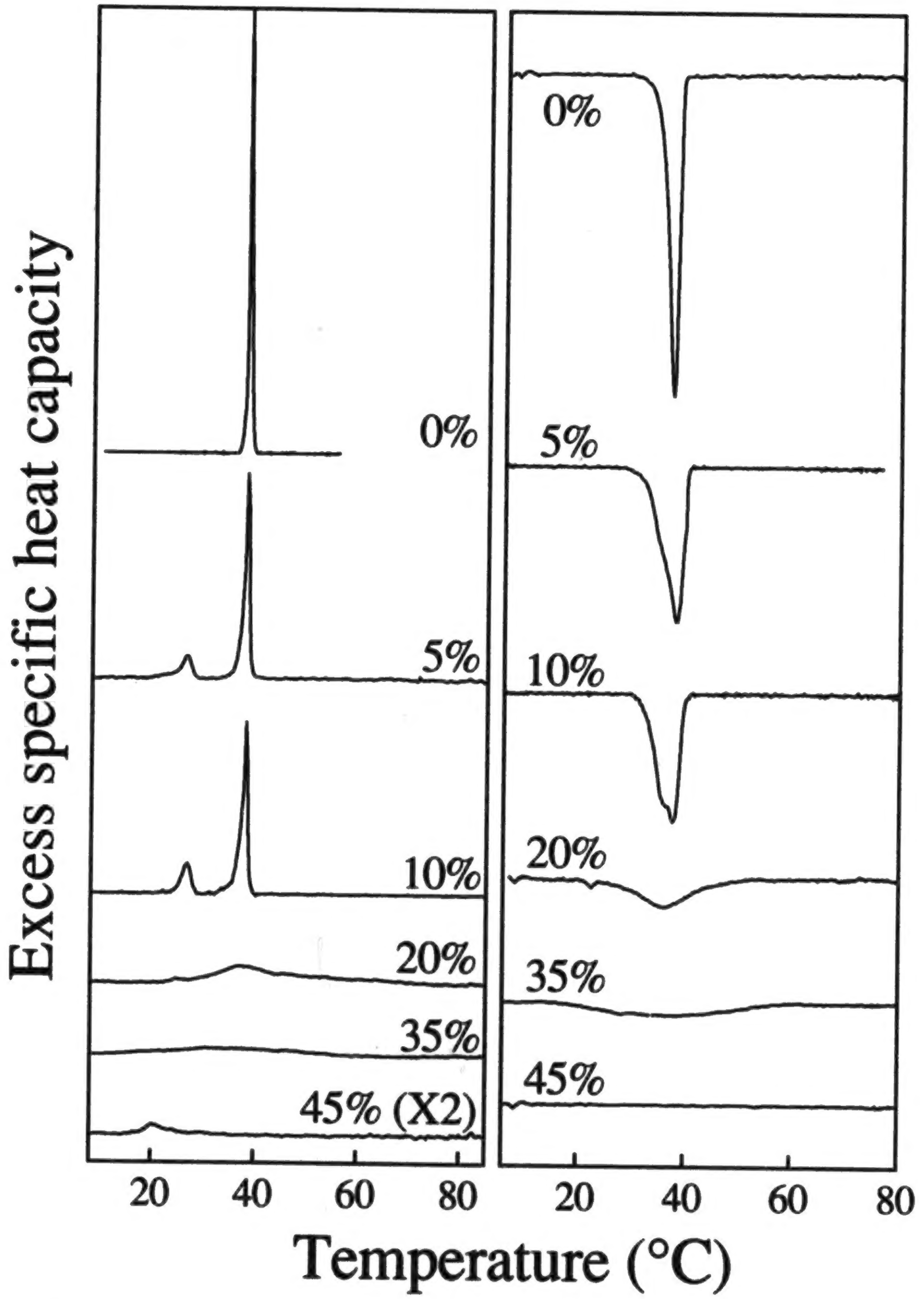

Excess specific heat capacity
0%
5%
10%
20%
35%
45% (X2)
0%
5%
10%
20%
35%
45%
20
40
60
80
20
40
60
80
Temperature (°C)

Figure VIII-2. Plot of the transition temperature of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DMPS mixtures obtained from both heating and cooling runs. Transition temperature values obtained upon heating are represented by solid symbols and upon cooling by open symbols. The L_β/L_α phase transition is represented by (heating ●) (cooling ○) and the L_c/L_β transition by (◆).

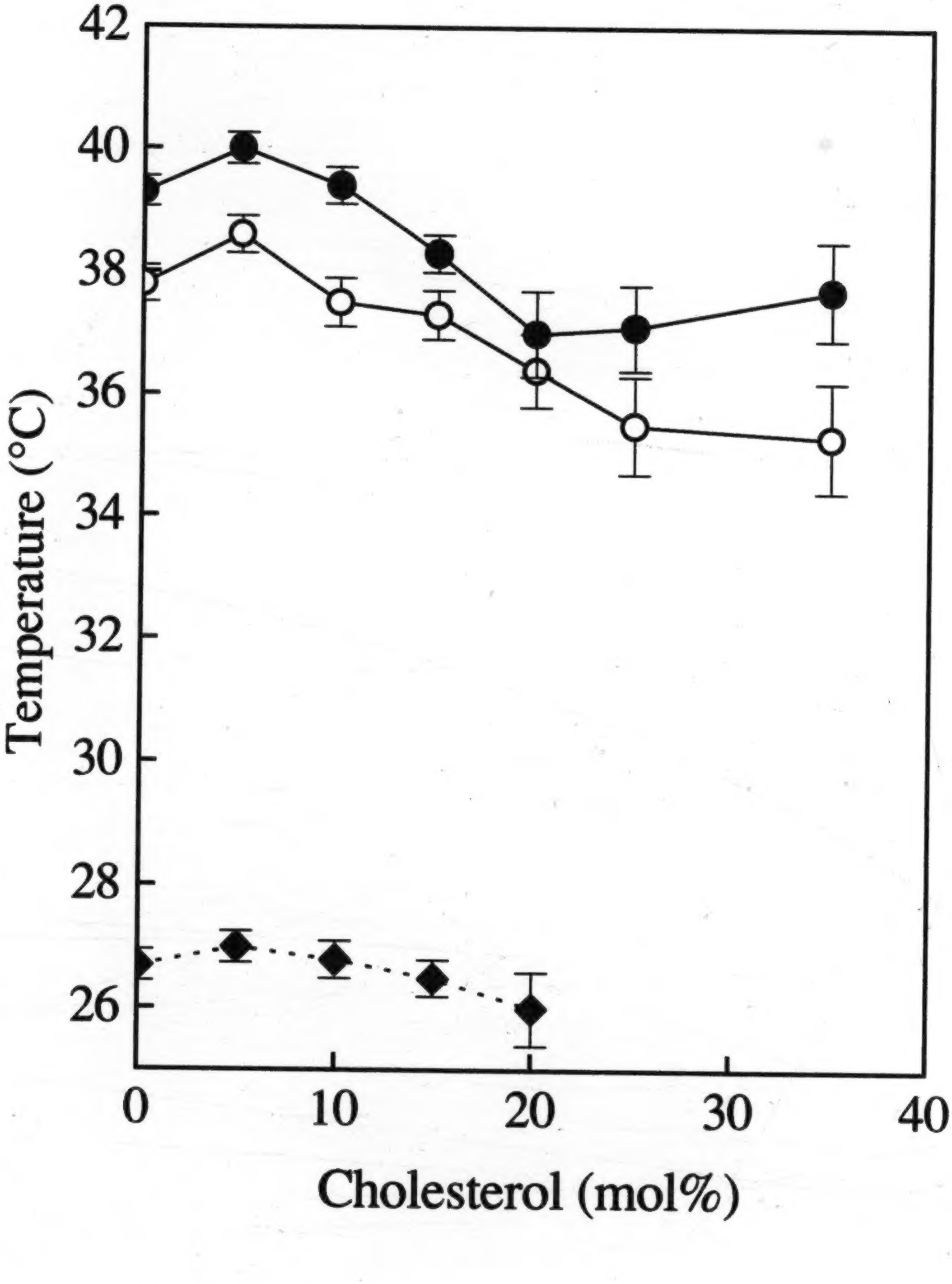

42
40
38
36
34
32
30
28
26
Temperature (°C)
0
10
20
30
40
Cholesterol (mol%)

Figure VIII-3. Plot of the enthalpy of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DMPS mixtures obtained from both heating and cooling runs. Enthalpy values obtained upon heating are represented by solid symbols and upon cooling by open symbols. The L_{β}/L_{α} phase transition is represented by (heating ●) (cooling ○) and the L_c/L_{β} transition by (◆).

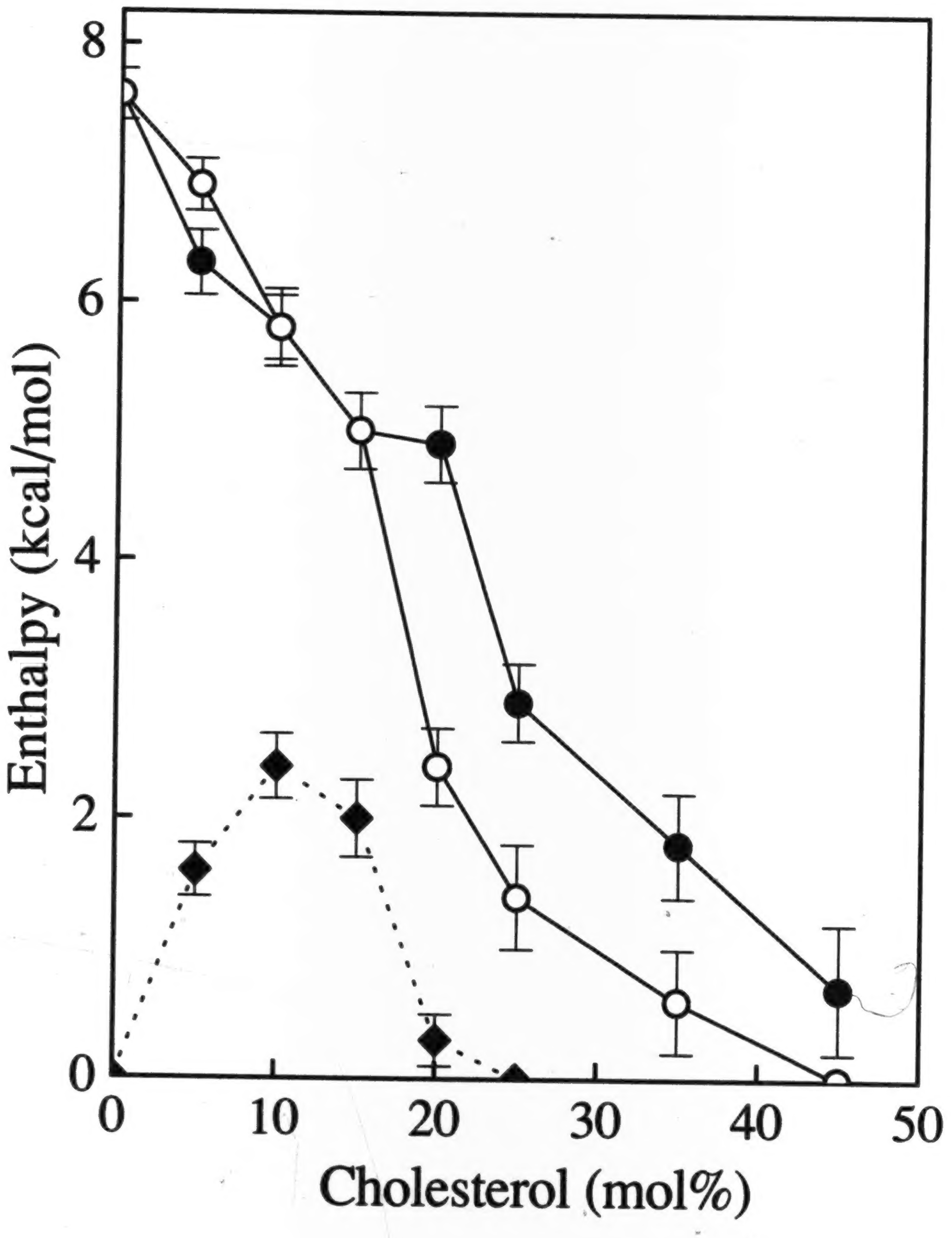

8
6
4
2
0
Enthalpy (kcal/mol)
0
10
20
30
40
50
Cholesterol (mol%)

Figure VIII-4. Representative heating (left panel) and cooling (right panel) thermograms of DPPS bilayers containing progressively increasing levels of cholesterol as indicated on the Figure. HS-DSC scans of DPPS bilayers containing 15 and 25 mol% cholesterol were also performed but are not shown. All endotherms shown were scanned at 10 °C/hr and are normalized for sample mass.

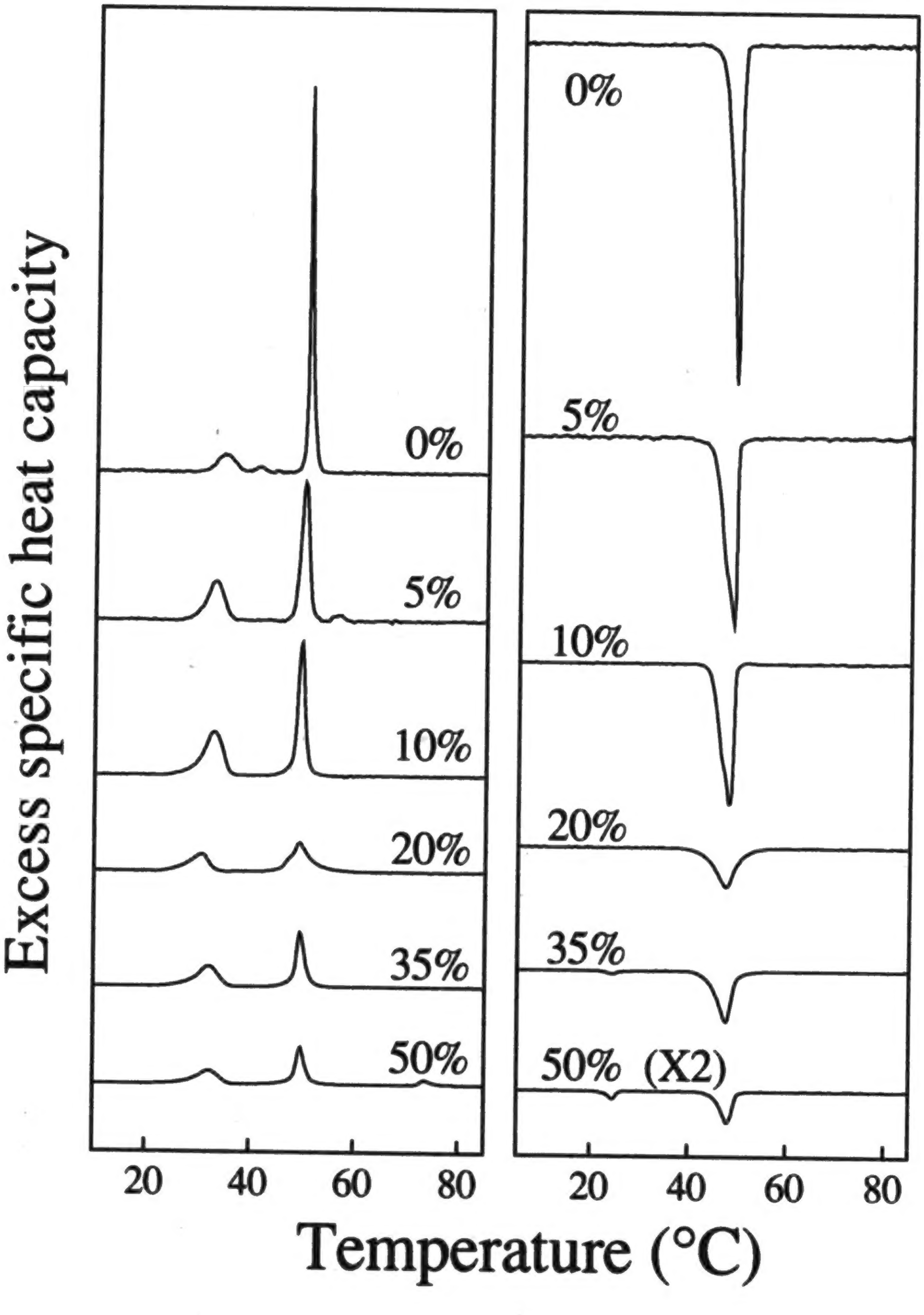

Excess specific heat capacity
0%
5%
10%
20%
35%
50%
0%
5%
10%
20%
35%
50% (X2)
20
40
60
80
20
40
60
80
Temperature (°C)

Figure VIII-5. Plots of the transition temperature of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DPPS mixtures obtained from both heating and cooling runs. Transition temperature values obtained upon heating are represented by solid symbols and upon cooling by open symbols. The L_β/L_α phase transition is represented by (heating ●) (cooling ○) and the L_c/L_β transition by (◆).

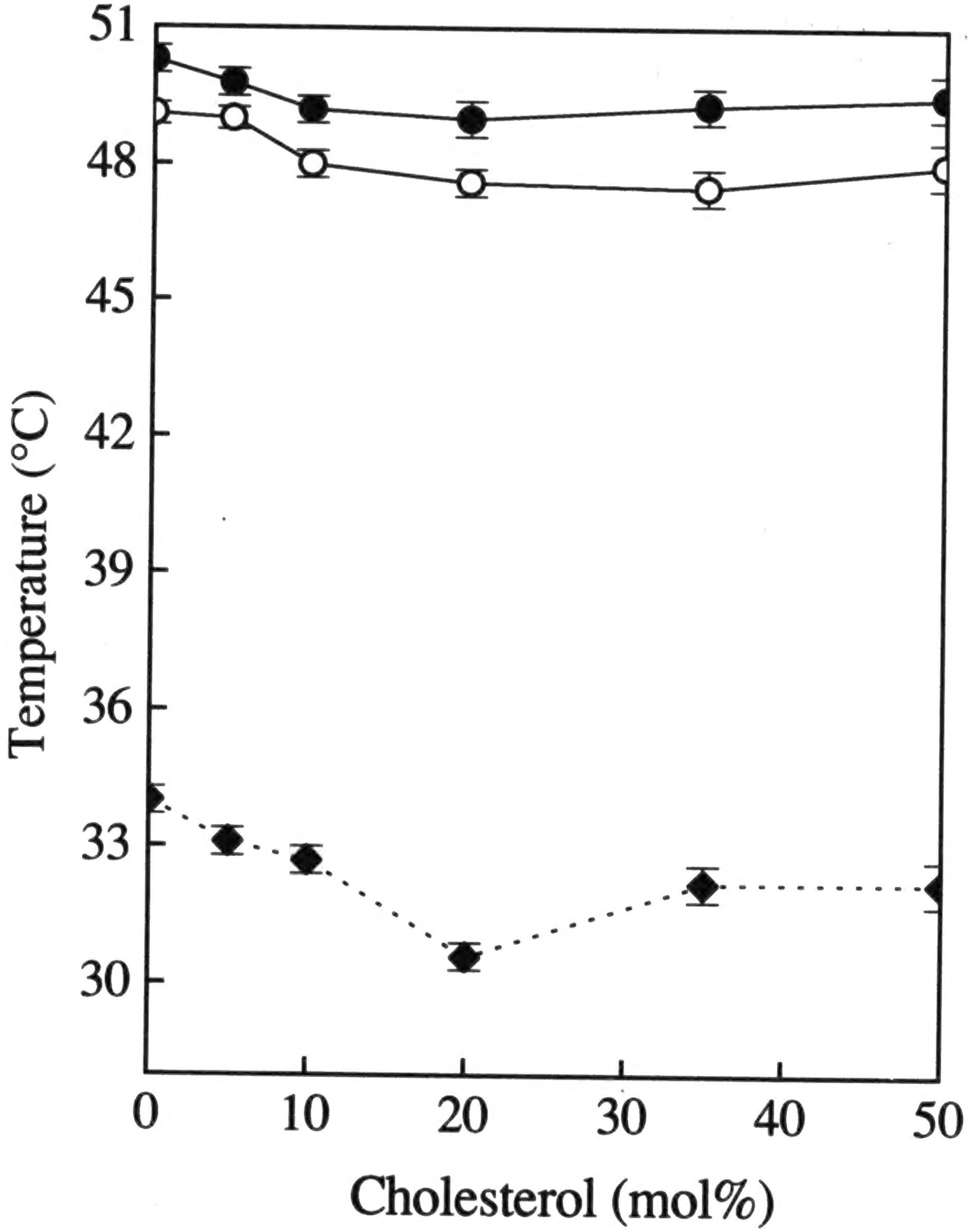

Temperature (°C)
51
48
45
42
39
36
33
30
0
10
20
30
40
50
Cholesterol (mol%)

Figure VIII-6. Plots of the enthalpy of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DPPS mixtures obtained from both heating and cooling runs. Enthalpy values obtained upon heating are represented by solid symbols and upon cooling by open symbols. The L_β/L_α phase transition is represented by (heating ●) (cooling ○) and the L_c/L_β transition by (◆).

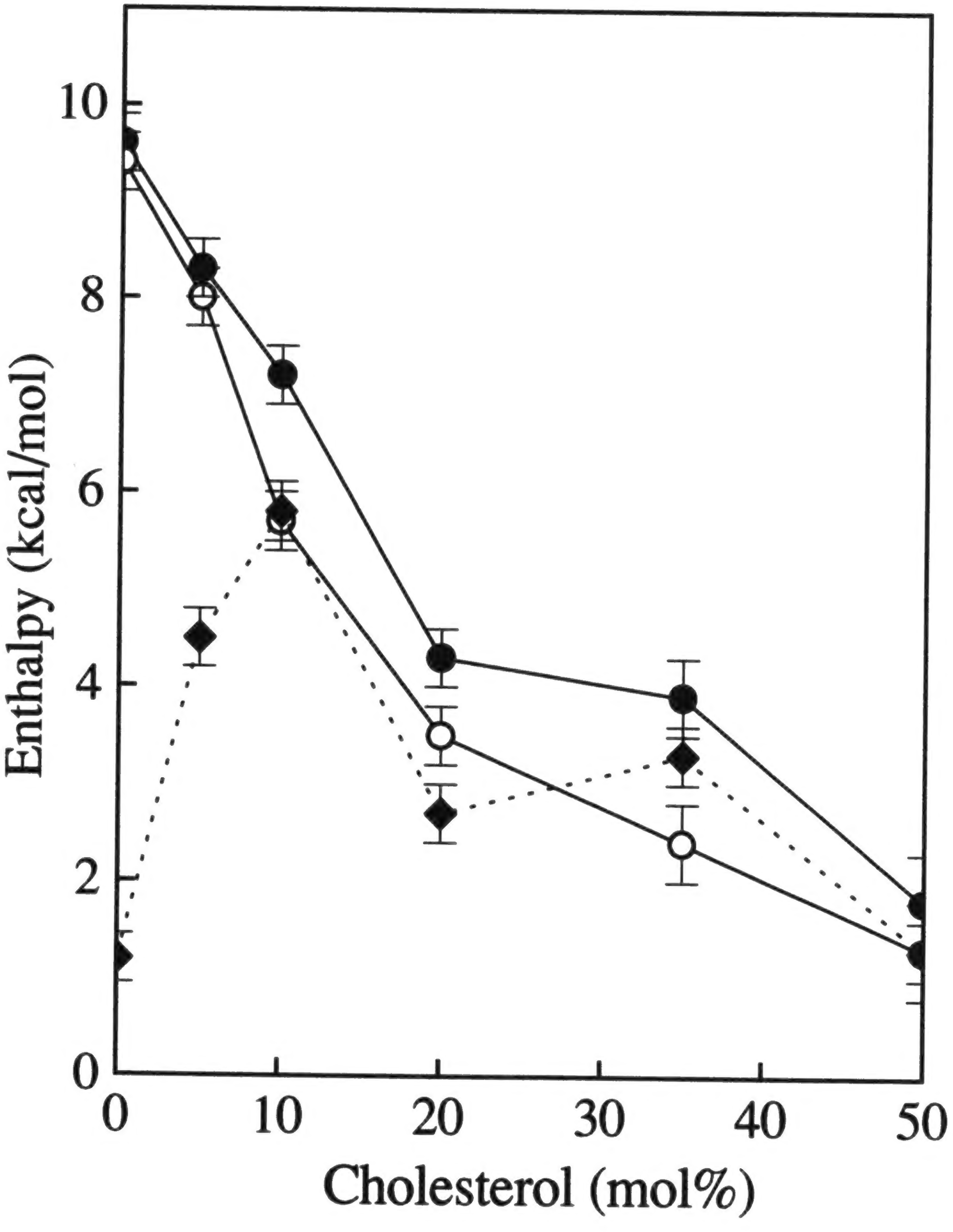

Enthalpy (kcal/mol)
0
2
4
6
8
10
Cholesterol (mol%)
0
10
20
30
40
50

Figure VIII-7. Representative heating (left panel) and cooling (right panel) endotherms of DSPS bilayers containing progressively increasing levels of cholesterol as indicated on the Figure. HS-DSC scans of DSPS bilayers containing 15 and 25 mol% cholesterol were also performed but are not shown. All endotherms shown were scanned at 10 °C/hr and are normalized for sample mass.

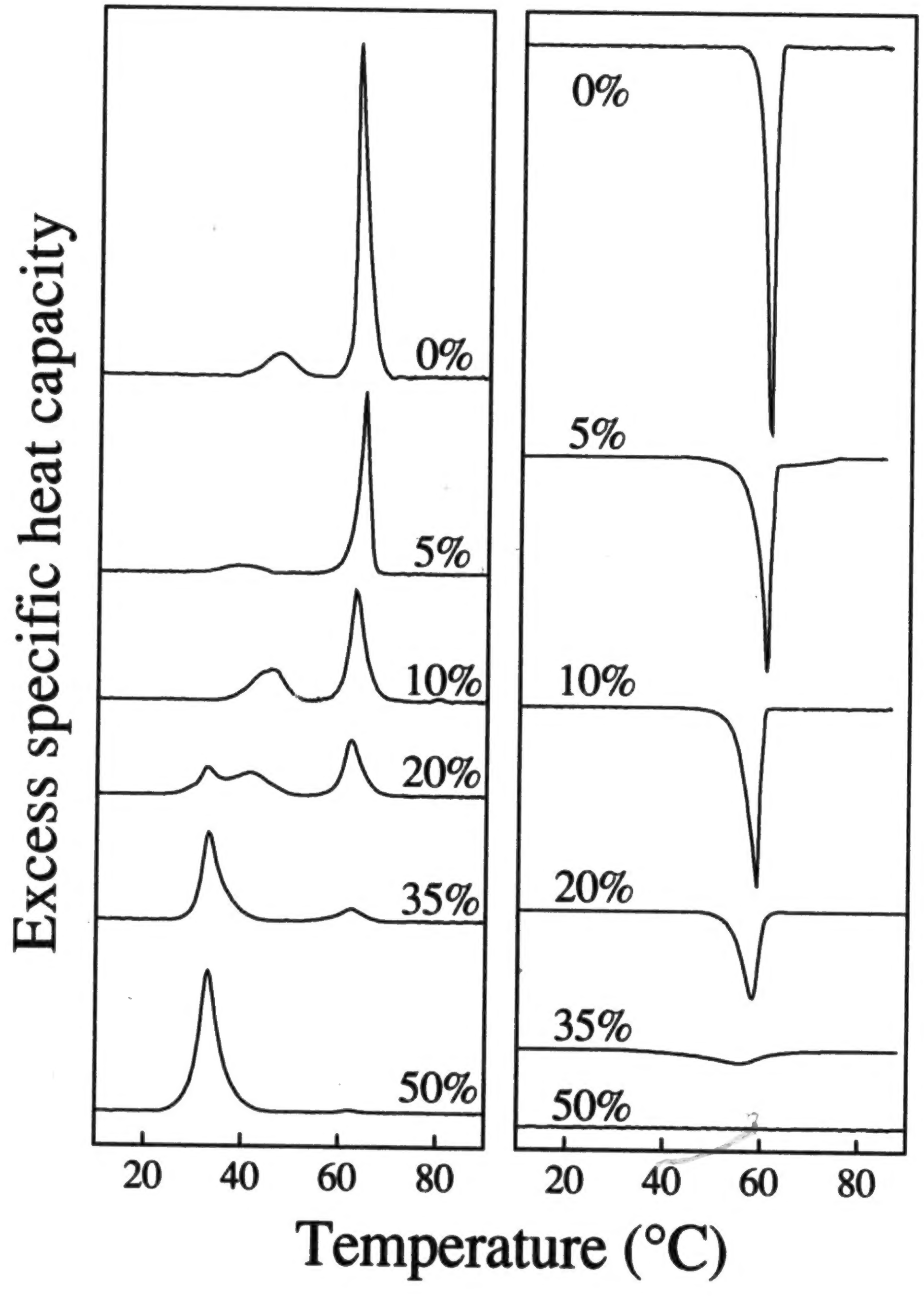

Excess specific heat capacity
0%
5%
10%
20%
35%
50%
0%
5%
10%
20%
35%
50%
20
40
60
80
20
40
60
80
Temperature (°C)

Figure VIII-8. Plots of the transition temperature of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DSPS mixtures obtained from both heating and cooling runs. Transition temperature values obtained upon heating are represented by solid symbols; the cooling mode by open symbols. The L_β/L_α phase transition is represented by (heating ●) (cooling ❍) and the L_c/L_β transition by (◆).

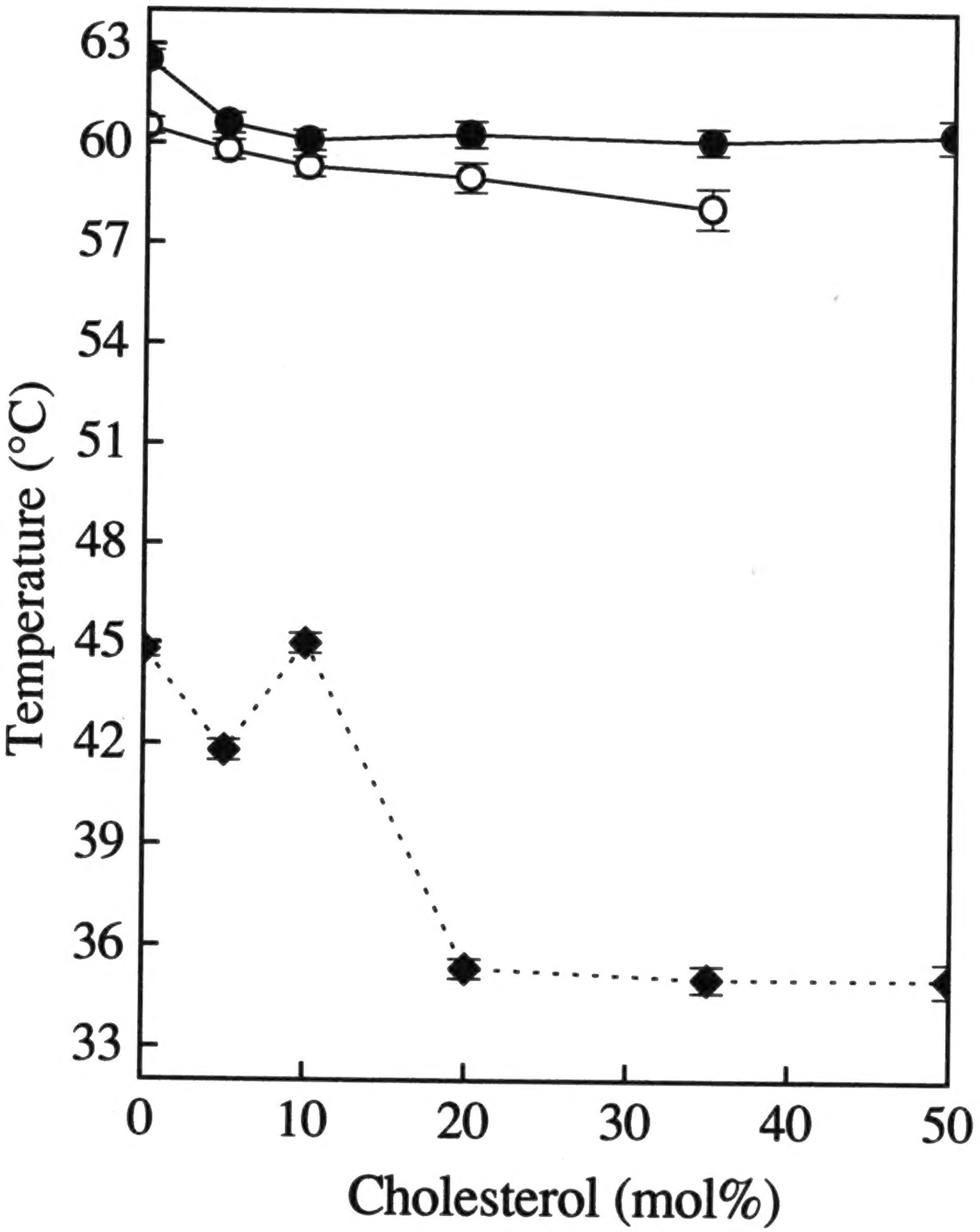

Temperature (°C)
63
60
57
54
51
48
45
42
39
36
33
0
10
20
30
40
50
Cholesterol (mol%)

Figure VIII-9. Plots of the enthalpy of the overall chain-melting transition as a function of increasing cholesterol concentration for cholesterol/DSPS mixtures obtained from both heating and cooling runs. Enthalpy values obtained upon heating are represented by solid symbols and upon cooling by open symbols. The L_β/L_α phase transition is represented by (heating ●) (cooling ○) and the L_c/L_β transition by (◆).

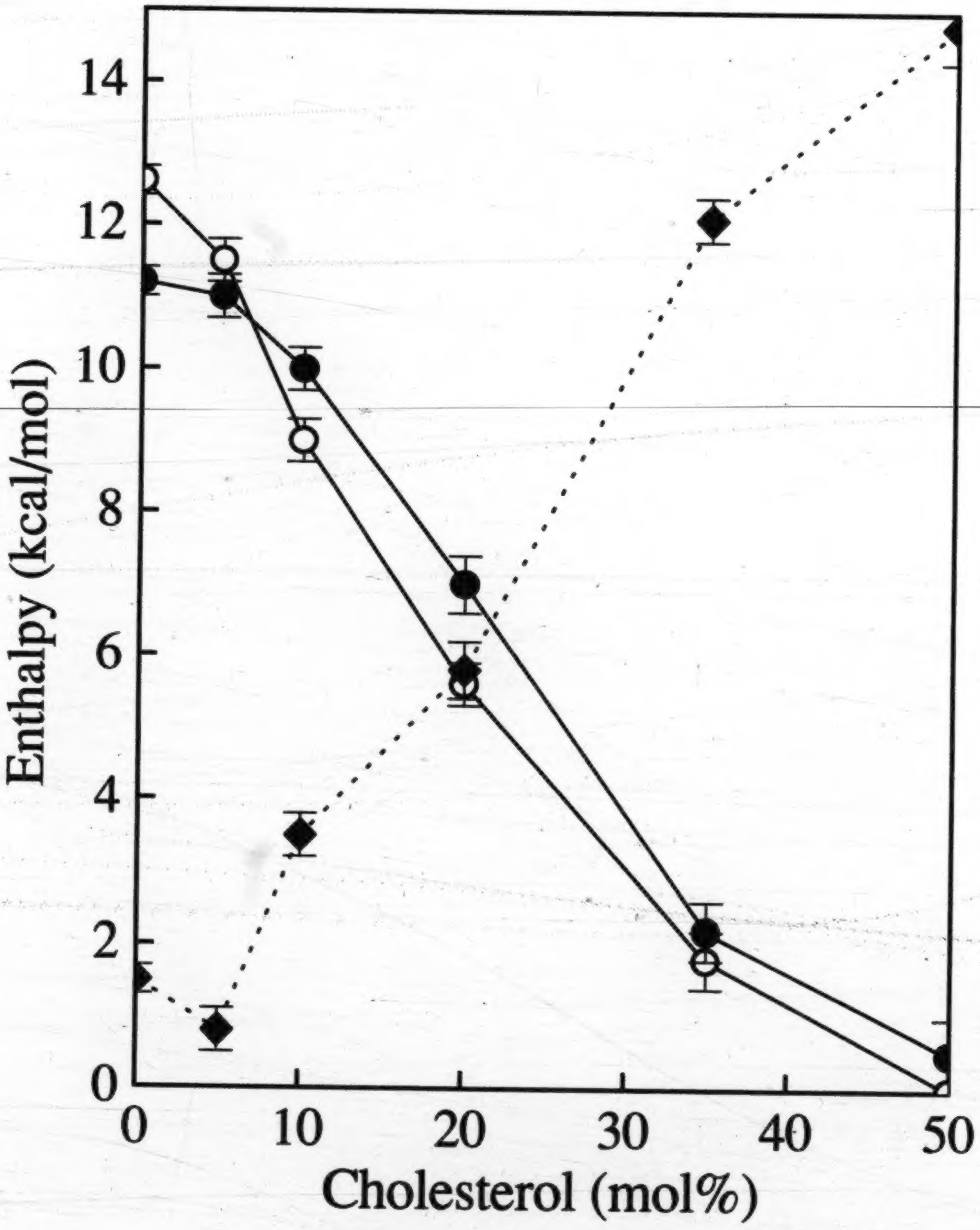

Enthalpy (kcal/mol)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
Cholesterol (mol%)

Figure VIII-10. Representative FTIR CH_2 stretching, C=O stretching and CH_2 scissoring absorption bands for the L_α, L_β, L_c1 and L_c2 phases of fully hydrated n-saturated diacyl PS bilayers, as indicated in the Figure. The left panel represents the CH_2 symmetric and asymmetric stretching bands, the middle panel the C=O stretching absorption bands and the right panel the CH_2 scissoring absorption bands.

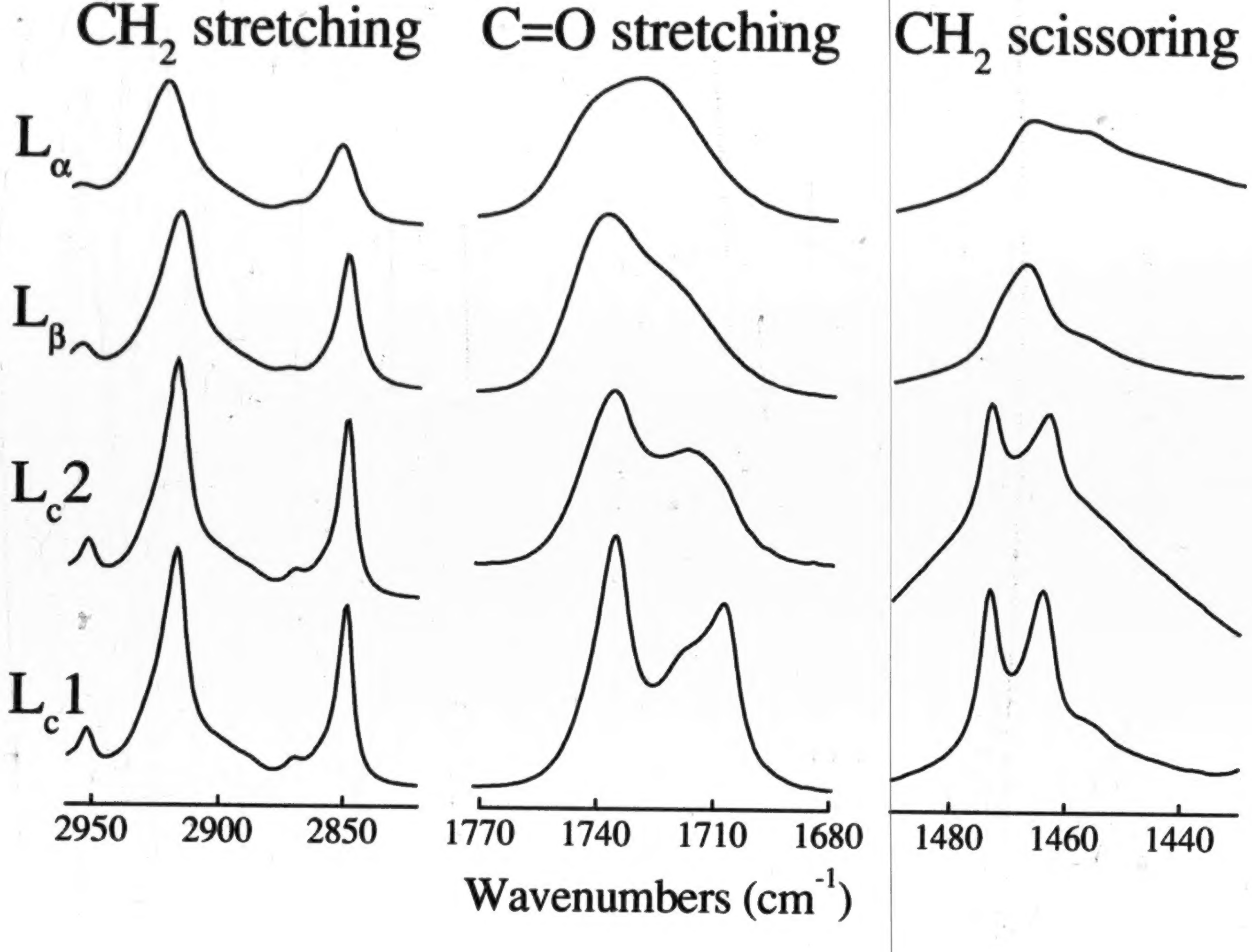

CH$_2$ stretching
C=O stretching
CH$_2$ scissoring
L$_\alpha$
L$_\beta$
L$_c$2
L$_c$1
2950
2900
2850
1770
1740
1710
1680
1480
1460
1440
Wavenumbers (cm^{-1})

Figure VIII-11. Representative FTIR C=O stretching and CH_2 scissoring absorption bands for DMPS bilayers containing cholesterol (see text for details). Shown in the left panel are the C=O stretching absorption bands and in the right panel the CH_2 scissoring absorption bands. Arrows indicate phase changes with increasing temperature.

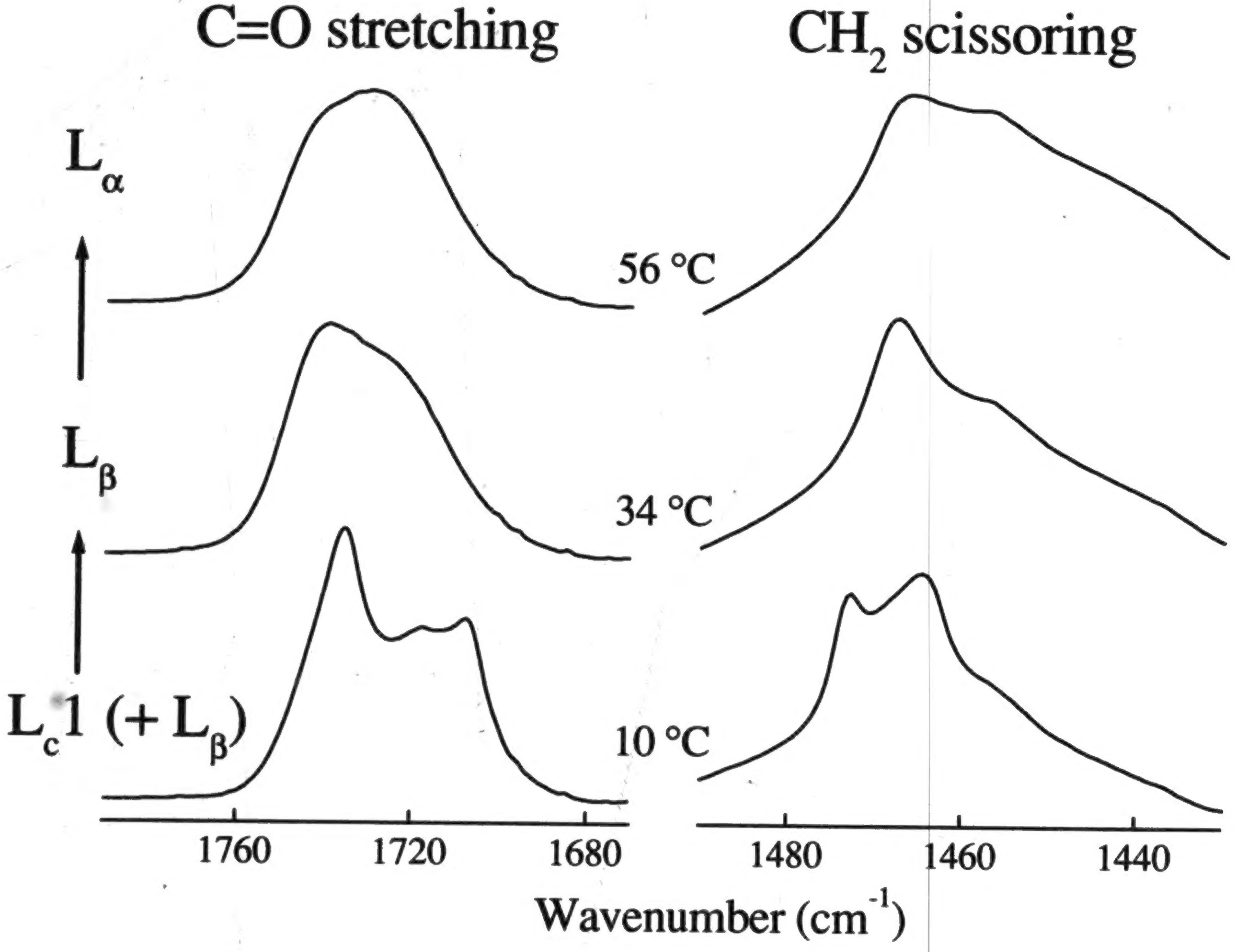

C=O stretching
CH$_2$ scissoring
L$_\alpha$
L$_\beta$
L$_c$1 (+ L$_\beta$)
56 °C
34 °C
10 °C
1760
1720
1680
1480
1460
1440
Wavenumber (cm^{-1})

Figure VIII-12. Representative FTIR C=O stretching and CH_2 scissoring absorption bands for DMPS, DPPS and DSPS bilayers containing various levels of cholesterol when monitored on cooling (see text for details). Shown in the left panel are the C=O stretching absorption bands and in the right panel the CH_2 scissoring absorption bands. Arrows indicate phase changes with decreasing temperature. All of the cholesterol/PS mixtures would eventually convert to the L_c1 or L_c2 phases if incubated at low temperatures, however, the time required to form crystalline phases varied with the level of cholesterol and the hydrocarbon chain length of the host PS bilayer.

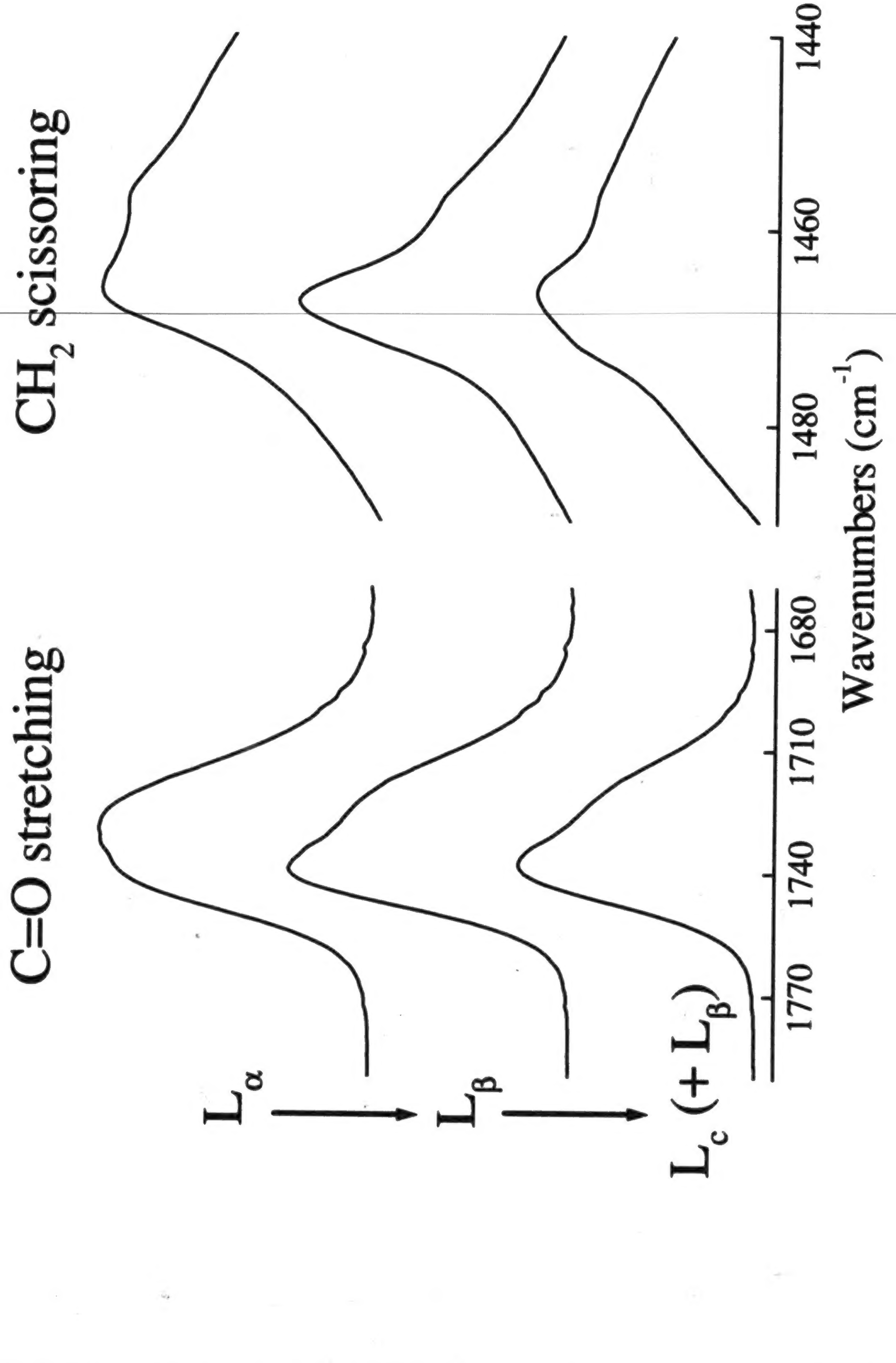

C=O stretching
CH$_2$ scissoring
L$_\alpha$
L$_\beta$
L$_c$ (+ L$_\beta$)
1770
1740
1710
1680
1480
1460
1440
Wavenumbers (cm^{-1})

Figure VIII-13. Representative FTIR C=O stretching and CH_2 scissoring absorption bands for DPPS bilayers containing cholesterol and DSPS bilayers containing from 0 to 25 mol% cholesterol (see text for details). Shown in the left panel are the C=O stretching absorption bands and in the right panel the CH_2 scissoring absorption bands. Arrows indicate phase changes with increasing temperature.

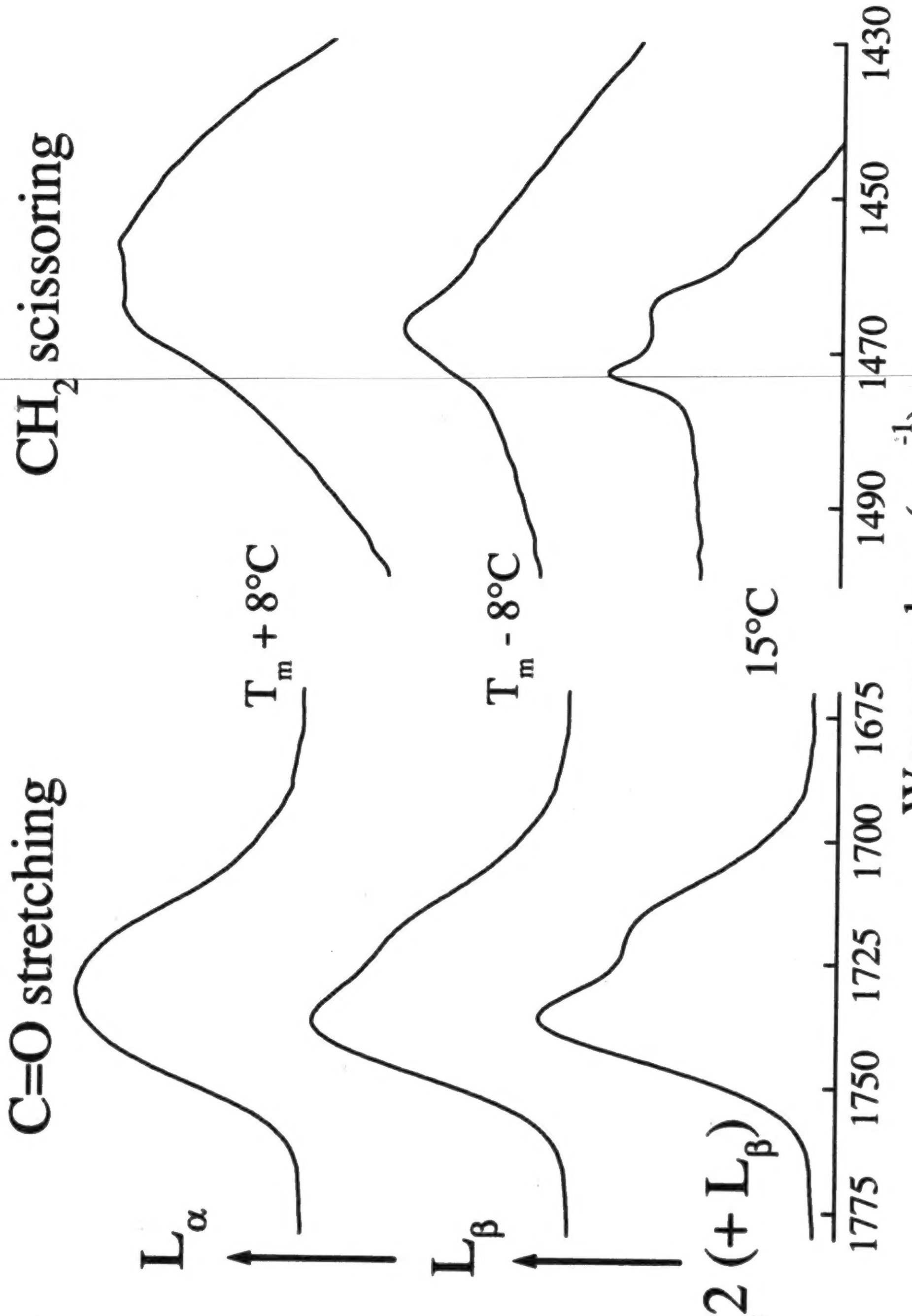
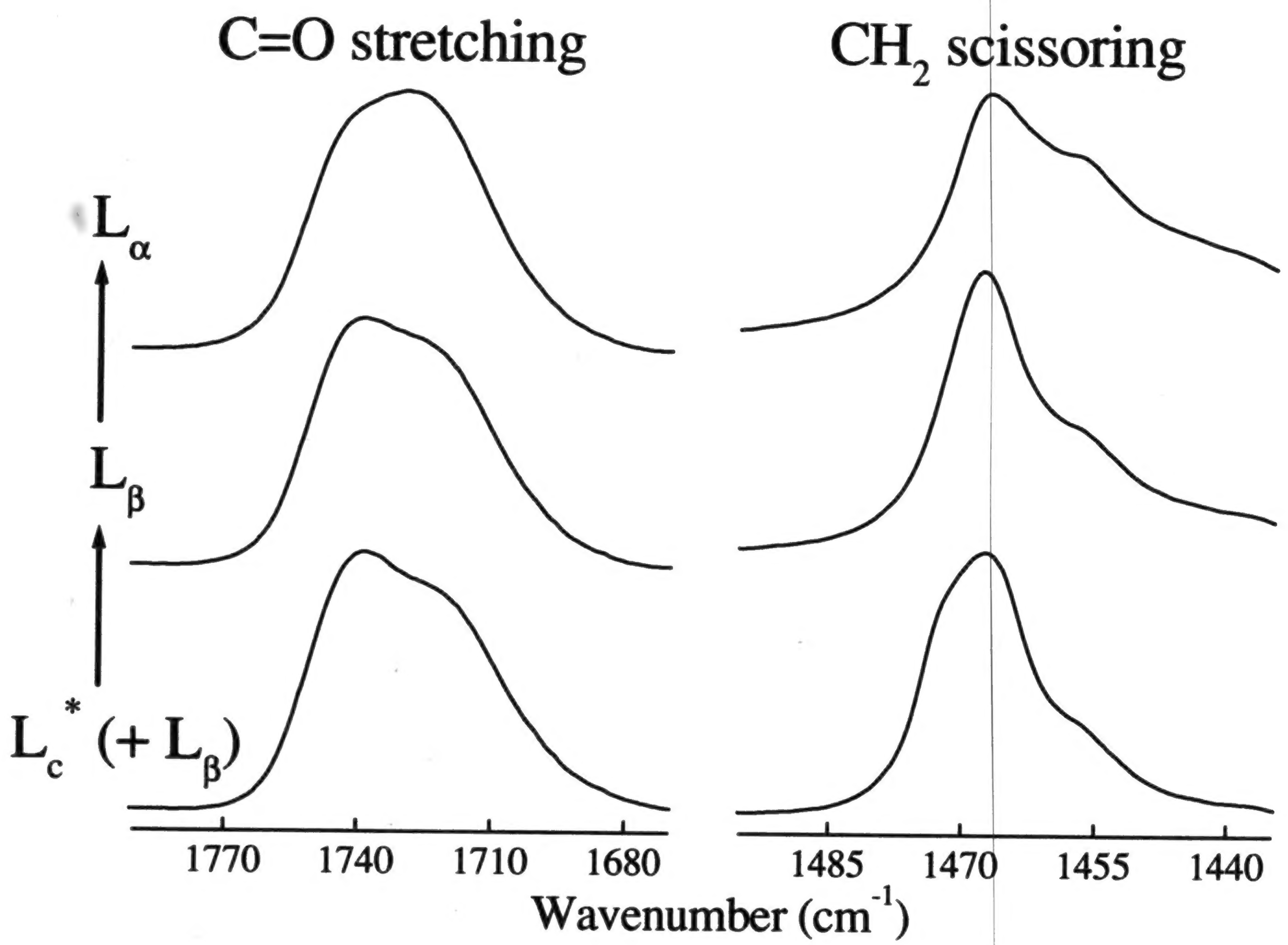

C=O stretching
CH$_2$ scissoring
L$_\alpha$
L$_\beta$
L$_c$2 (+L$_\beta$)
T$_m$ + 8°C
T$_m$ - 8°C
15°C
1775
1750
1725
1700
1675
1490
1470
1450
1430
Wavenumbers (cm^{-1})

Figure VIII-14. Representative FTIR C=O stretching and CH_2 scissoring absorption bands for DSPS bilayers containing cholesterol levels exceeding 35 mol% (see text for details). Shown in the left panel are the C=O stretching absorption bands and in the right panel the CH_2 scissoring absorption bands. Arrows indicate phase changes with increasing temperature.


C=O stretching
CH$_2$ scissoring
L$_\alpha$
L$_\beta$
L$_c$* (+ L$_\beta$)
1770
1740
1710
1680
1485
1470
1455
1440
Wavenumber (cm^{-1})

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CHAPTER IX. DIFFERENTIAL SCANNING CALORIMETRIC STUDIES OF THE INTERACTION OF CHOLESTEROL WITH DISTEAROYL AND DIELAIDOYL PHOSPHATIDYLCHOLINE, PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLSERINE[9]

INTRODUCTION

The occurrence of substantial quantities of cholesterol (or of a structurally similar sterol) in the plasma membranes of virtually all eukaryotic cells has prompted many investigations into the role of cholesterol in the structure and function of cell membranes (for reviews, see Dahl and Dahl, 1988; Yeagle, 1988; McElhaney, 1992a,b,c). Although cholesterol and related sterols appear to have several different functions in eukaryotic cells, one of its primary and apparently essential roles is as a modulator of the physical properties of the phospholipid bilayer of the plasma membrane. Thus numerous studies of the interactions of cholesterol with single-component phospholipid model membranes have been carried out using many different physical techniques (for reviews, see Demel and de Kruijff, 1976; Razin and Rottem, 1978; Yeagle, 1988; Finean, 1990; McElhaney, 1992a,b,c; McMullen and McElhaney, 1996). There is now a consensus that the incorporation of increasing levels of cholesterol broadens and eventually eliminates altogether the cooperative gel/liquid-crystalline phase transition of the host lipid bilayer. In addition, cholesterol increases the degree of orientational order and reduces the rate of motion of the phospholipid hydrocarbon chains in the biologically relevant liquid-crystalline state, condensing the bilayer laterally, increasing its mechanical strength and decreasing its permeability, while maintaining the relatively high rates of lateral and rotational diffusion characteristic of the fluid phospholipid bilayers. Although much fewer in number, similar studies of the interactions of cholesterol with the complex mixtures of lipids found in natural membranes seem to yield similar results. However, despite an impressive body of experimental data derived from both model and biological membranes, our detailed understanding of the molecular basis for the effects of cholesterol on phospholipid bilayers remains incomplete.

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[9] A version of this chapter has been submitted for publication to *Biochemistry*. McMullen, T.P.W., and McElhaney, R. N. (1996).

The vast majority of studies of cholesterol/phospholipid interactions have utilized binary mixtures of cholesterol with a PC containing two identical linear saturated hydrocarbon chains, especially DPPC. Although it has been suggested that the thermotropic phase behavior of cholesterol/DPPC mixtures is generally representative of all molecular species of PC's (Ipsen *et al.*, 1987; Thewalt and Bloom, 1992; Linseisen *et al.*, 1993), in fact considerable evidence exists that there are significant variations in such behavior, depending on the fatty acid composition of the host PC bilayer. For example, the miscibility of cholesterol, or various side chain truncated cholesterol analogues, with various PC molecular species, and the effect of the incorporated sterol on the phase transition temperature and organization of the host PC bilayer, has been shown to be markedly dependent on hydrocarbon chain length (McMullen *et al.*, 1993, 1994, 1995; McMullen and McElhaney, 1996; Vilchèze *et al.*, 1996). Moreover, the thermotropic phase behavior of binary mixtures of cholesterol or cholesterol analogues with PC's containing one or two unsaturated hydrocarbon chains can be quite different from that of binary mixtures of cholesterol with linear saturated PC's (Davis and Keough, 1983; Keough *et al.*, 1989; Kariel *et al.*, 1991; Vilchèze *et al.*, 1996). As well, the thermotropic phase behavior of mixtures of cholesterol with synthetic or naturally occurring phospholipids such as PE's (Blume, 1980; Epand and Bottega, 1987; Cheetham *et al.*, 1989; McMullen *et al.*, 1996a), PS's (Bach, 1984; Wachtel and Bach, 1987; Wachtel *et al.*, 1991; Bach *et al.*, 1992) or PG's (Borochov *et al.*, 1995), as well as with the neutral glyceroglycolipids MGDG and DGDG from the *Acholeplasma laidlawii* B membrane (McMullen *et al.*, 1996b), can differ significantly from that of binary mixtures of cholesterol with the homologous PCs. In addition, the interactions of cholesterol with phospho- and glycosphingolipids can vary both with respect to each other and to the related glycerophospho- and glyceroglycolipids (Bach, 1984). Thus, the detailed nature of cholesterol-lipid interactions can vary appreciably with the structure of the polar headgroup and interfacial region, and with the structure and length of the hydrocarbon chains, of the phospho- or glycolipid molecules which make up the host lipid bilayer (see McMullen and McElhaney, 1996).

The PC's and PE's are generally the two major zwitterionic phospholipids of eukaryotic membranes and the PS's are the major anionic phospholipids. In the present comparative study, we have investigated the effects of cholesterol incorporation on the thermotropic phase behavior of aqueous dispersions of these three important phospholipid classes by high-sensitivity DSC, in order to assess the effect of variations in lipid polar headgroup structure on the nature of cholesterol/phospholipid interactions. Within each phospholipid class we have also compared the distearoyl and dielaidoyl molecular species in order to assess the influence of *trans*-unsaturation on the interactions of cholesterol with the host phospholipid bilayer. (The effects of variations in hydrocarbon chain length on the thermotropic phase behavior of cholesterol/PC (McMullen *et al.*, 1993), cholesterol/PE (McMullen *et al.*, 1996a) and cholesterol/PS (McMullen *et al.*, 1996c) are reported elsewhere.) We do indeed find that variations in phospholipid polar headgroup structure and the degree of unsaturation of the fatty acyl chains can both have significant effects on the thermotropic phase behavior of cholesterol/phospholipid binary mixtures in water.

MATERIALS AND METHODS

The PC's, PE's and PS's used in these experiments were purchased from Avanti Polar Lipids (Alabaster, AB) and checked for purity by TLC using chloroform/methanol/ammonia (50:50:4, by vol.) (PC's and PE's) or chloroform/methanol/glacial acetic acid/water (60:40:10:4, by volume) (PS's) as the developing solvent followed by spraying with 2% K_2CrO_4 in 60% sulfuric acid and charring. Each phospholipid gave a single spot on the developed TLC plates. The cholesterol was also purchased from Avanti Polar Lipids and recrystallized from ethanol before use. We found that the best procedure for producing homogenous mixtures of phospholipid and cholesterol required chloroform/methanol (2:1, vol/vol) stock solutions or lyophilization from benzene which, after mixing, were heated to approximately 40 to 50

°C under N_2 to remove the solvent, then dried under vacuum for at least 18 hours. When carefully followed, both protocols provide fully reproducible thermograms (see also McMullen *et al.*, submitted). Failure to lyophilize from benzene or to heat the chloroform/methanol phospholipid/cholesterol mixture would often require repeated heating and cooling DSC runs upon hydration to achieve consistent thermogram profiles.

For the HS-DSC experiments, the dried phospholipid/cholesterol mixtures were dispersed and suspended in a buffer containing 50 mM Tris, 100 mM NaCl and 10 mM EDTA (pH 7.4), heated to approximately 10-20°C above the phase transition of the mixture, and then vortexed for at least 30 minutes to give a multilamellar suspension. When we examined variations in buffer (Tris and phosphate) and ionic strength (NaCl 0.100 to 0.400 M) on the thermotropic phase behavior of these phospholipid/cholesterol mixtures, we observed only small shifts in the transition temperature and no changes in the qualitative behavior of the sample. For all of the HS-DSC samples containing cholesterol, the mixtures were hydrated and suspended as detailed, then stored for 1 day at 2 °C before the HS-DSC experiment. The HS-DSC thermograms for the cholesterol/PS suspensions were recorded with a Hart 7701 high-sensitivity differential scanning calorimeter (Provo, UT). The scan rates used were 10 °C/hr unless otherwise noted. In addition, the amount of phospholipid used for the DSC analyses was progressively increased from 0.5 mg for pure phospholipid bilayers to 20 mg for phospholipid samples containing 45 or 50 mol% cholesterol. We have shown previously that this protocol is required to accurately monitor the broad, low-enthalpy phase transitions observed at higher cholesterol concentrations (McMullen *et al.*, 1993). The Hart calorimeter was calibrated using solid standards from Hart Scientific, as well as aqueous lipid samples synthetically prepared and purified within this laboratory using methods previously shown to provide highly pure samples (Lewis *et al.*, 1985). Sample runs were repeated at least three times to ensure reproducibility. Low temperature-annealed samples typically involved holding the temperature at 2 °C anywhere from 2 to 48 hours, depending on the sample, but may also involve more complicated regimes as detailed in Lewis and McElhaney (1993). For all mixtures phospholipid and cholesterol degradation was monitored by TLC and no degradation products were observed. Moreover, sequential HS-DSC runs were completely reproducible, supporting the absence of chemical degradation in our samples after HS-DSC or spectroscopic analysis. The analysis and the decomposition of the HS-DSC endotherms was done using Microcal's Origin (Northhampton, MA) and DA-2 software.

RESULTS

Thermotropic phase behavior of cholesterol/phosphatidylcholine mixtures.

Representative high-sensitivity DSC heating thermograms of DSPC and DEPC, alone and in the presence of various amounts of cholesterol, are presented in Figure IX-1. In the absence of cholesterol, DSPC and DEPC bilayers exhibit two endotherms on heating, a lower temperature, lower enthalpy pretransition and a higher temperature and higher enthalpy main transition. The pretransition arises from the conversion of a lamellar gel (L_{β}') phase to the rippled gel (P_{β}') phase and the main transition from a conversion of the P_{β}' phase to the lamellar liquid-crystalline (L_{α}) phase. These transitions are fully reversible on cooling (results not shown). As expected, both the pretransition (9.5 °C) and main transition (12 °C) temperatures (Figure IX-2) of DEPC are considerably lower than for DSPC (50.5 °C and 54.0 °C, respectively) due to the presence of a *trans*-double bond in each hydrocarbon chain of the former and the enthalpy of the main transition of DEPC (7.9 kcal/mol) is also somewhat lower that that of DSPC (9.3 kcal/mol) (Figure IX-3). The pretransition of both PC's is abolished upon the incorporation of 5 mol% or more of cholesterol.

The DSC heating scans in Figure IX-1 also illustrate the overall effect of the incorporation of increasing quantities of cholesterol on the main phase transition of DSPC and DEPC. At cholesterol concentrations of less than about 20 mol%, the asymmetric DSC endotherms clearly consist of two overlapping components (see McMullen *et al.*, 1993). As illustrated in Figure IX-2, the phase transition temperature of the sharp component decreases moderately with increasing cholesterol incorporation and its cooperativity decreases moderately (Figure IX-1) for both cholesterol/PC mixtures. Moreover, the enthalpy of the sharp component decreases markedly with increasing cholesterol concentration, becoming zero between 20 and 25 mol% cholesterol (Figure IX-3). In contrast, the phase transition temperature of the broad component of the DSC endotherm either decreases more markedly than that of the sharp component (DSPC) or actually increases slightly (DEPC) (Figure IX-2), and in both cases the cooperativity of the broad transition decreases markedly as cholesterol concentration increases. As a result of the difference in the relative temperature shifts of the sharp and broad components for cholesterol/DSPC and cholesterol/DEPC mixtures, the multi-component nature of the chain-melting transition of cholesterol/DEPC mixtures is more evident. The enthalpy of the broad component of both the DSPC and DEPC endotherms first increases with increasing cholesterol concentration, reaching a local maximum at 20-25 mol%, and then decreases again to zero at 50 mol% cholesterol. Thus in both cases the total enthalpy of the PC chain melting phase transition enthalpy decreases more or less linearly from 0 to 50 mol% cholesterol (Figure IX-3). It is important to note that, except for the direction of the shift in the temperature of the broad component, the thermotropic phase behavior of DSPC and DEPC are influenced in exactly the same way by the incorporation of various quantities of cholesterol. One should also note that the DSC endotherms obtained upon heating and the DSC exotherms obtained upon cooling are essentially identical (data not presented), indicating that cholesterol is equally and fully miscible in both the gel and liquid-crystalline phases of these compounds.

Thermotropic phase behavior of cholesterol/phosphatidylethanolamine mixtures.

Representative DSC heating and cooling thermograms of DSPE and DEPE, alone and in the presence of various amounts of cholesterol, are presented in Figures IX-4 and IX-5, respectively. In the absence of cholesterol, DSPE and DEPE bilayers exhibit a single, highly energetic and cooperative chain-melting (L_β/L_α) phase transition. The chain-melting phase transition of DSPE is centered at 74.5 °C and has an enthalpy value of 11.2 kcal/mol, while that of DEPE is centered at 37.5 °C and the phase transition enthalpy is 8.0 kcal/mol. In addition, DEPE bilayers also exhibit a higher temperature, lower enthalpy lamellar liquid-crystalline to reversed hexagonal (L_α/H_{II}) phase transition centered at 57.3

°C. The main phase transition of both compounds is reversible on heating and cooling as is the L_α/H_{II} phase transition of DEPE, albeit with some hysteresis for the latter process.

The influence of cholesterol incorporation on the thermotropic phase behavior of aqueous dispersions of DSPE is illustrated in Figure IX-4. The effect of the incorporation of increasing quantities of cholesterol into DSPE bilayers is to progressively decrease the temperature (Figure IX-6), enthalpy (Figure IX-7) and cooperativity (Figure IX-4) of the main phase transition in both the heating and cooling DSC modes. Note that in contrast to the cholesterol/PC binary mixtures, the DSC thermograms are not readily resolvable into sharp and broad components at low cholesterol concentrations and multiple components are clearly present at cholesterol concentrations of 20 mol% and higher. Cholesterol also produces a much greater depression of the phase transition temperature and enthalpy at high cholesterol concentrations when monitored in the cooling mode. Moreover, the enthalpy of the main phase transition of DSPE is not reduced to zero at 50 mol% cholesterol on either heating and cooling modes. These results indicate that cholesterol is not fully miscible with DSPE bilayers in either the gel or liquid-crystalline states, but is considerably more miscible in the latter than the former.

For cholesterol/DEPE mixtures, the incorporation of cholesterol progressively reduces the temperature (Figure IX-6), enthalpy (Figure IX-7) and cooperativity (Figure IX-5) of the lamellar chain-melting transition such that by 50 mol% cholesterol, this phase transition is virtually abolished on both heating and cooling. Conversely, the effect of cholesterol on the non-lamellar L_α/H_{II} transition is to markedly increase both the temperature (Figure IX-6) and enthalpy (Figure IX-7) of this transition while only moderately affecting its cooperativity (Figure IX-5).

Thermotropic phase behavior of cholesterol/phosphatidylserine mixtures.

Representative DSC heating and cooling thermograms of DSPS and DEPS, alone and in the presence of various amounts of cholesterol, are presented in Figures IX-8 and IX-9, respectively. In the absence of cholesterol, both DSPS and DEPS bilayers exhibit a single, highly energetic and cooperative chain-melting (L_β/L_α) phase transition. For DSPS, the chain-melting phase transition occurs at 62 °C and has an enthalpy value of 12.4 kcal/mol, while for DEPS the phase transition is centered at 25 °C and has an enthalpy value of 8.0 kcal/mol. The main phase transition of both compounds is reversible on heating and cooling. In addition, DSPS bilayers also exhibit a lower temperature (44.5 °C) and lower enthalpy (1.7 kcal/mol) L_c/L_β phase transition when monitored on heating.

The influence of cholesterol incorporation on the thermotropic phase behavior and of aqueous dispersions of DSPS is illustrated in Figure IX-8. The effect of the incorporation of increasing quantities of cholesterol into DSPS bilayers is to progressively decrease the temperature (Figure IX-10), enthalpy (Figure IX-11) and cooperativity (Figure IX-8) of the main phase transition in both the heating and cooling modes. Note that in contrast to the cholesterol/PC binary mixtures, the DSC thermograms are not readily resolvable into sharp and broad components at low cholesterol concentrations and multiple components are clearly present at cholesterol concentrations of 10 mol% and higher. From 20 to 50 mol% cholesterol, an additional, low-temperature endotherm is apparent in these mixtures which increases in size with increasing cholesterol levels. This endotherm results from cholesterol dissolution in the DSPS bilayer and from the conversion from the L_c to the L_β phase (data not presented). The relative sizes of these components, one centered at approximately 45°C and the other at 35 °C, also varies with the cholesterol content of the mixture, the low-temperature component dominating at cholesterol levels exceeding 20 mol%. By 50 mol% cholesterol the chain-melting transition at 61 °C is almost abolished on heating, but the low temperature transition becomes progressively more energetic. Thus, increasing the level of cholesterol actually increases the overall enthalpy of the DSPS phase transitions (Figure IX-11). In contrast, when monitored in the cooling mode, these mixtures exhibit only the chain-melting phase transition at 59 °C and cholesterol produces a much greater depression of the phase transition temperature (Figure IX-10) and enthalpy (Figure IX-11) at high cholesterol concentrations. Moreover, the enthalpy of the main phase transition of DSPS is reduced to zero at 50 mol% cholesterol in the cooling mode. These results indicate that cholesterol is almost fully miscible with DSPS bilayers in the gel state and completely miscible in DSPS bilayers in the liquid-crystalline state.

When monitored on heating, the incorporation of cholesterol into DEPS bilayers (Figure IX-9) results in small but progressive decreases in the temperature (Figure IX-10) but relatively large decreases in the enthalpy (Figure IX-11) and cooperativity (Figure IX-9) of the chain-melting phase transition. Interestingly, the chain-melting phase transition clearly exhibits at least two, and sometimes three, different components. These three components do not exhibit the same cholesterol-dependent shifts in temperature, enthalpy or cooperativity as those observed with the cholesterol-rich or -poor phospholipid endotherms in the corresponding cholesterol/DEPC mixtures. By 50 mol% cholesterol, the phase transition of DEPS bilayers is almost, but not completely, abolished. When monitored on cooling, the phase behavior of cholesterol/DEPS mixtures is virtually identical to that observed on heating. Thus cholesterol is almost completely miscible with DEPS in both the gel and liquid-crystalline states.

DISCUSSION

The effect of cholesterol on the thermotropic phase behavior of aqueous dispersions of the three eukaryotic phospholipids studied here clearly depends on both the structure of the polar headgroup and the degree of unsaturation of the host phospholipid molecule when the effective lengths of the hydrocarbon chains are comparable. For example, when comparing distearoyl molecular species of these three phospholipids in the heating mode, the miscibility of cholesterol decreases in the order DSPC>DSPS>DSPE, as measured by the decreasing ability of high concentrations of cholesterol to abolish the chain-melting phase transition. Similarly, when comparing the distearoyl with the dielaidoyl molecular species of any single phospholipid, where differences in the miscibility of cholesterol are observed (as for DSPE and DEPE), the dielaidoyl phospholipid generally exhibits the greater miscibility. Also, where differences in the miscibility of cholesterol of the gel and liquid-crystalline states are observed (as for DSPE and DEPE), cholesterol is less miscible in the gel state.

The results discussed above can be explained, at least qualitatively, by considering the strength of the intermolecular interactions characteristic of the various phospholipid molecular species studied, as manifested, for example, in their relative gel to liquid-crystalline phase transition temperatures. The relative phase transition temperatures of the three phospholipid classes studied here increase in the order PC<PS<PE and, for any given phospholipid class, the dielaidoyl species undergoes the chain-melting phase transition at a considerably lower temperature than does the distearoyl molecular species. The high phase transition temperature of the PE molecular species is due to the strong attractive electrostatic and hydrogen bonding interactions characteristic of the polar headgroup of this phospholipid relative to PS and especially to PC (Hauser *et al.*, 1981; Boggs, 1980, 1986, 1987; Hauser *et al.*, 1988; Lewis and McElhaney, 1993). Although these differences are most pronounced in the gel state, the higher gel to liquid-crystalline phase transition temperature of the PE's is also manifest as a greater degree of order in the liquid-crystalline state, even at comparable reduced temperatures, at least relative to the PC's (Lafleur *et al.*, 1990; Senak *et al.*, 1991; Davies *et al.*, 1992; Tuchtenhagen *et al.*, 1994). We therefore conclude that the miscibility of cholesterol with any particular phospholipid will be inversely related to the degree of order or the tightness of packing characteristic of that phospholipid at a given temperature and phase state. Interestingly, this also seems to be true of the miscibility of hydrophobic peptides transmembrane peptides with PC and PE (Zhang *et al.*, 1992a,b; 1995a,b) and PS (unpublished observations from this laboratory) bilayers. Although other, more subtle and specific interactions may occur in particular phospholipid/cholesterol binary systems, it seems that, in general, the relative strength of phospholipid-cholesterol interactions, as manifest in the ability of cholesterol to maximize phospholipid-cholesterol and to minimize cholesterol-cholesterol interactions, is determined primarily by the strength of phospholipid-phospholipid interactions in most phospholipid bilayer systems. Phospholipids with strong intermolecular interactions with one another tend to exclude cholesterol from the bilayer above certain critical concentrations. This finding seems also to hold for the MGDG, DGDG and PG components of the *A. laidlawii* membrane, where the higher-melting neutral glycolipids exhibit a more limited ability to mix with cholesterol (Monck *et al.*,

1993; McMullen *et al.*, 1996b). The tendency for phospholipid-cholesterol lateral phase separation can be reduced by decreasing the strength of the intermolecular phospholipid-phospholipid interactions. This can be accomplished by decreasing the phospholipid phase transition temperature by changes in polar headgroup structure, by introducing hydrocarbon chain unsaturation, or by increasing the temperature of the system. Finally, it is important to note that we do not observe the presence of solid-phase cholesterol in any of the cholesterol/phospholipid mixtures examined here, in contrast to the results of some of the prior studies of cholesterol/PE and cholesterol/PS mixtures (Bach, 1984; Bach and Wachtel, 1987; Wachtel *et al.*, 1991; Bach *et al.*, 1992). Differences in the hydrocarbon chain structure and length of the PE and PS molecular species used in these prior studies and the present one may be partly responsible for this discrepancy. However, the care which we took in the present work to ensure that all cholesterol/phospholipid mixtures were well mixed and fully hydrated may also account for our findings.

This study, as well as a similar study of the interaction of cholesterol with the individual phospho- and glycolipids of the *A. laidlawii* membrane (McMullen *et al.*, 1996c), have again demonstrated that the strength and nature of lipid/cholesterol interactions can vary markedly with the detailed chemical structure of the lipid molecule, and thus that the cholesterol/PC systems are not an adequate model for cholesterol interactions in biological membranes, with their generally very complex lipid compositions.

Figure IX-1. Representative DSC heating scans of aqueous dispersions of DSPC (left panel) and DEPC (right panel) bilayers containing various concentrations of cholesterol. The scans are corrected for mass and scan rate. Cooling scans are essentially identical and are not reproduced here.

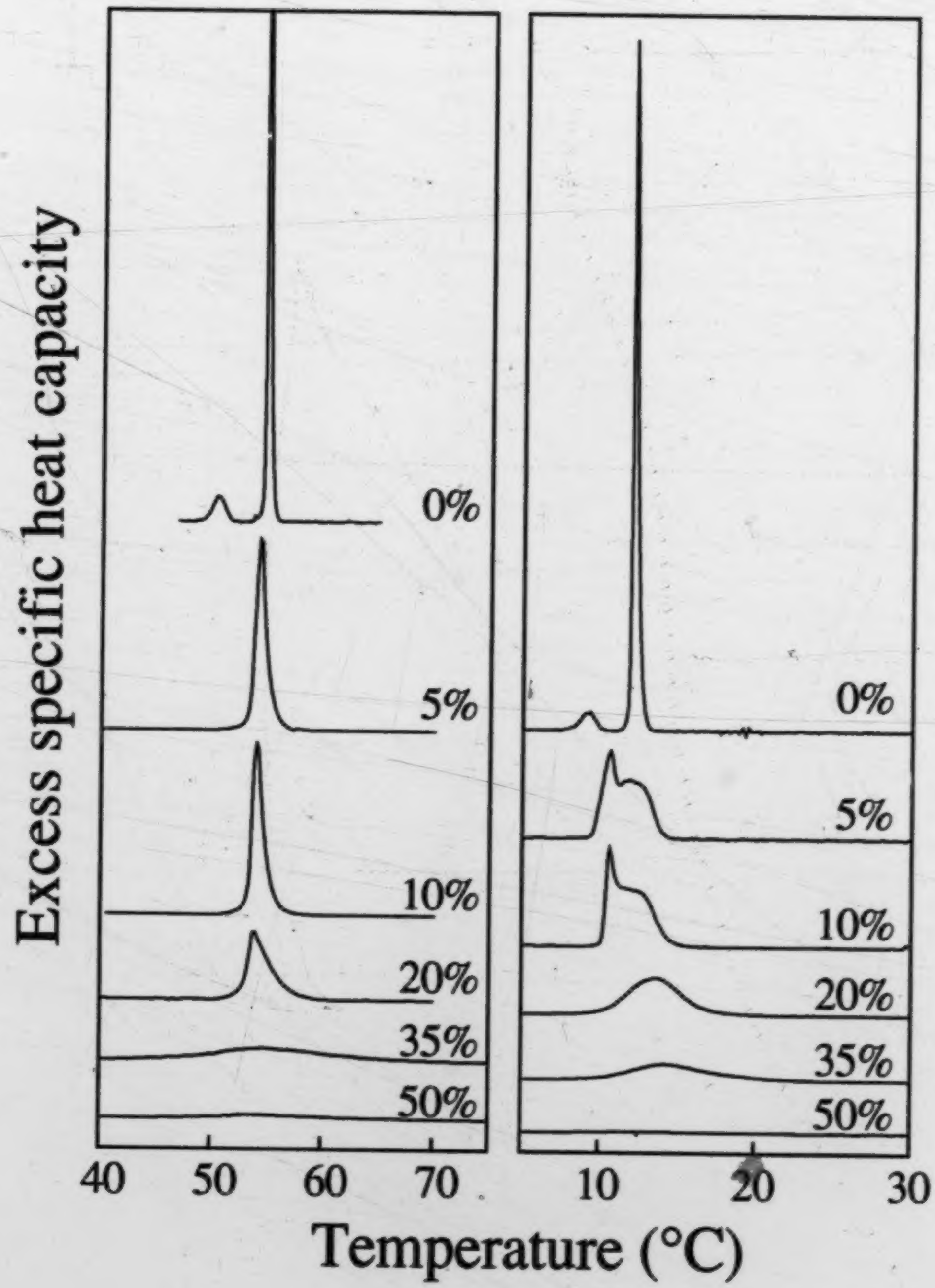

Excess specific heat capacity
0%
5%
10%
20%
35%
50%
0%
5%
10%
20%
35%
50%
40
50
60
70
10
20
30
Temperature (°C)

Figure IX-2. Representative plots of the temperatures of the DSPC (heating ●) (cooling ○) and DEPC (heating ■) (cooling ❑) chain melting transition. Transition temperatures were corrected for differences in heating scan rates between the various samples analyzed.

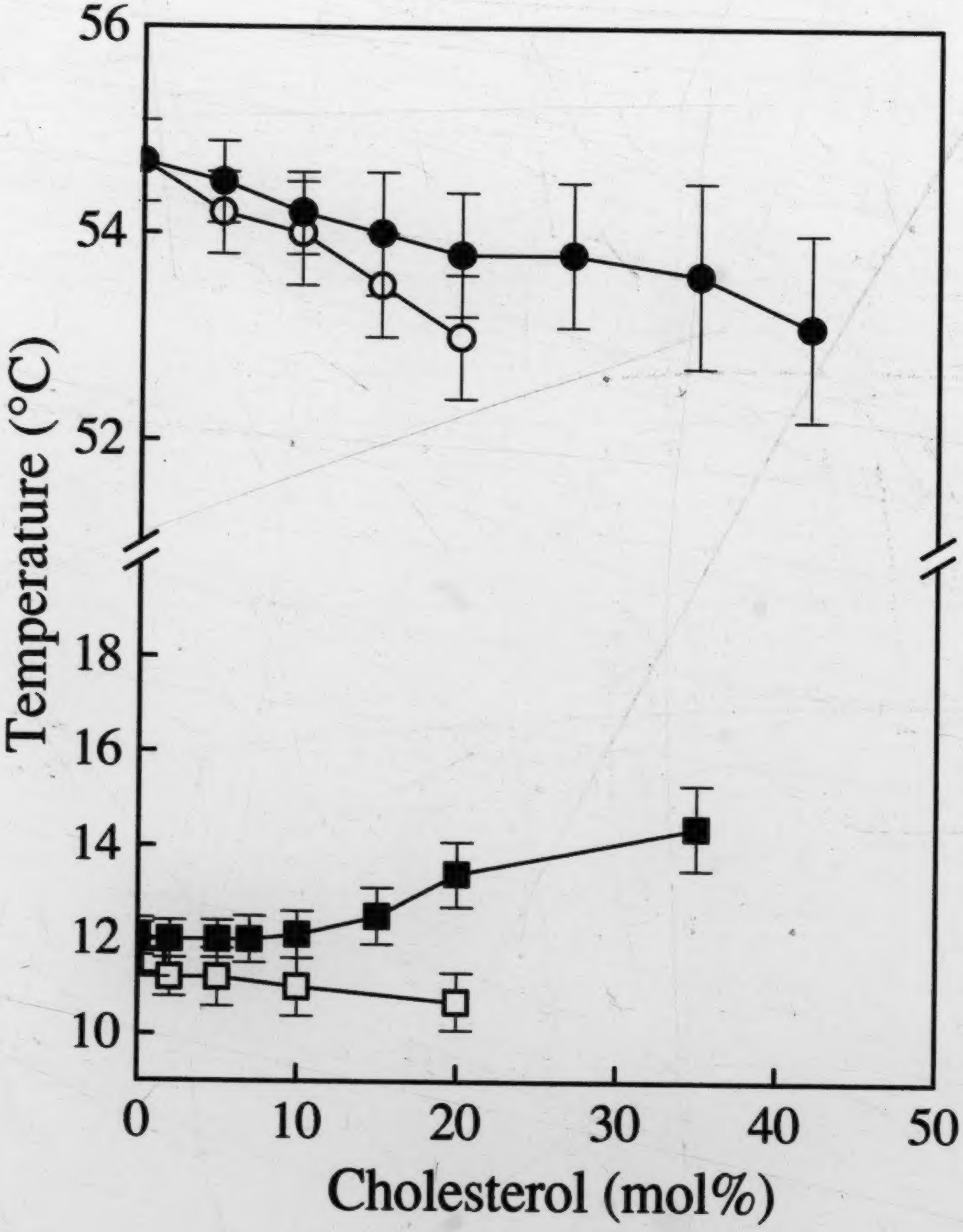

56
54
52
18
16
14
12
10
Temperature (°C)
0
10
20
30
40
50
Cholesterol (mol%)

Figure IX-3. Representative plots of the total enthalpy for the DSPC (●) and DEPC (■) chain-melting transitions as a function of cholesterol concentration in the heating mode (the enthalpy values in the cooling mode are essentially identical). The enthalpy of the sharp and broad components of the DSPC (○) and DEPC (❑) chain-melting transitions are also shown.

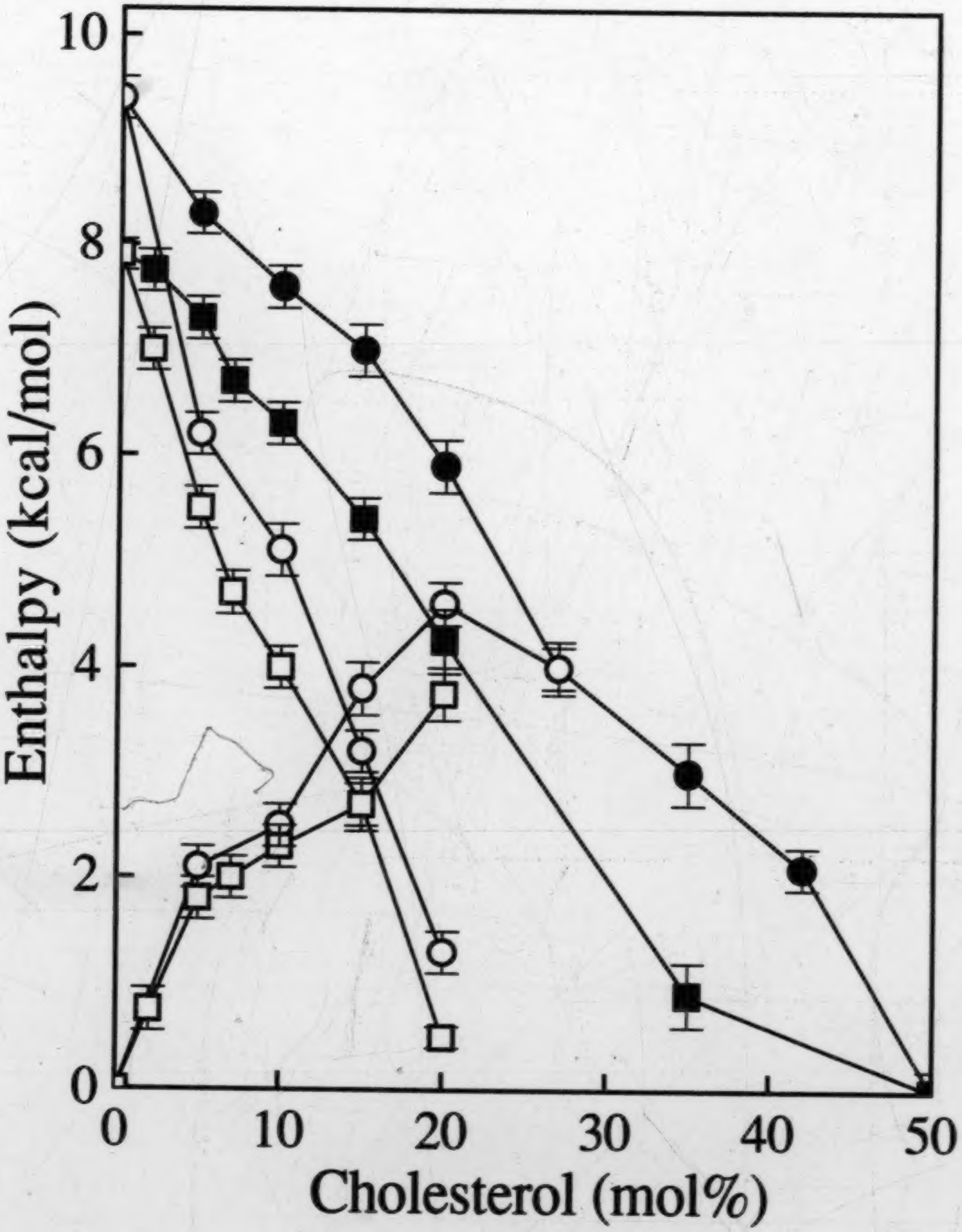

Enthalpy (kcal/mol)
Cholesterol (mol%)
0
2
4
6
8
10
0
10
20
30
40
50

Figure IX-4. Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DSPE bilayers containing various concentrations of cholesterol. The scans are adjusted for mass and scan rate.

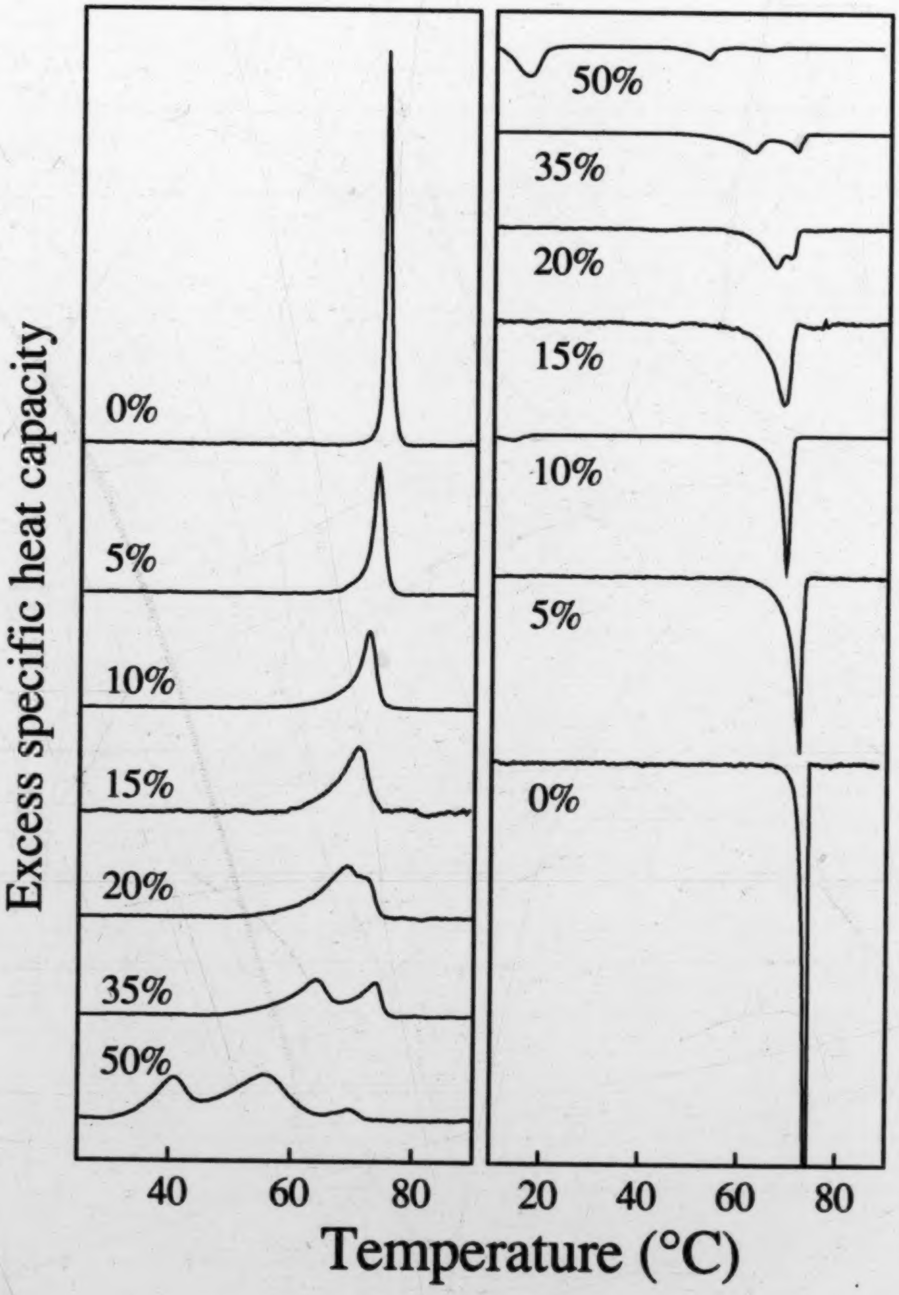

Excess specific heat capacity
0%
5%
10%
15%
20%
35%
50%
50%
35%
20%
15%
10%
5%
0%
40
60
80
20
40
60
80
Temperature (°C)

Figure IX-5. Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DEPE bilayers containing various concentrations of cholesterol. The scans are adjusted for mass and scan rate.

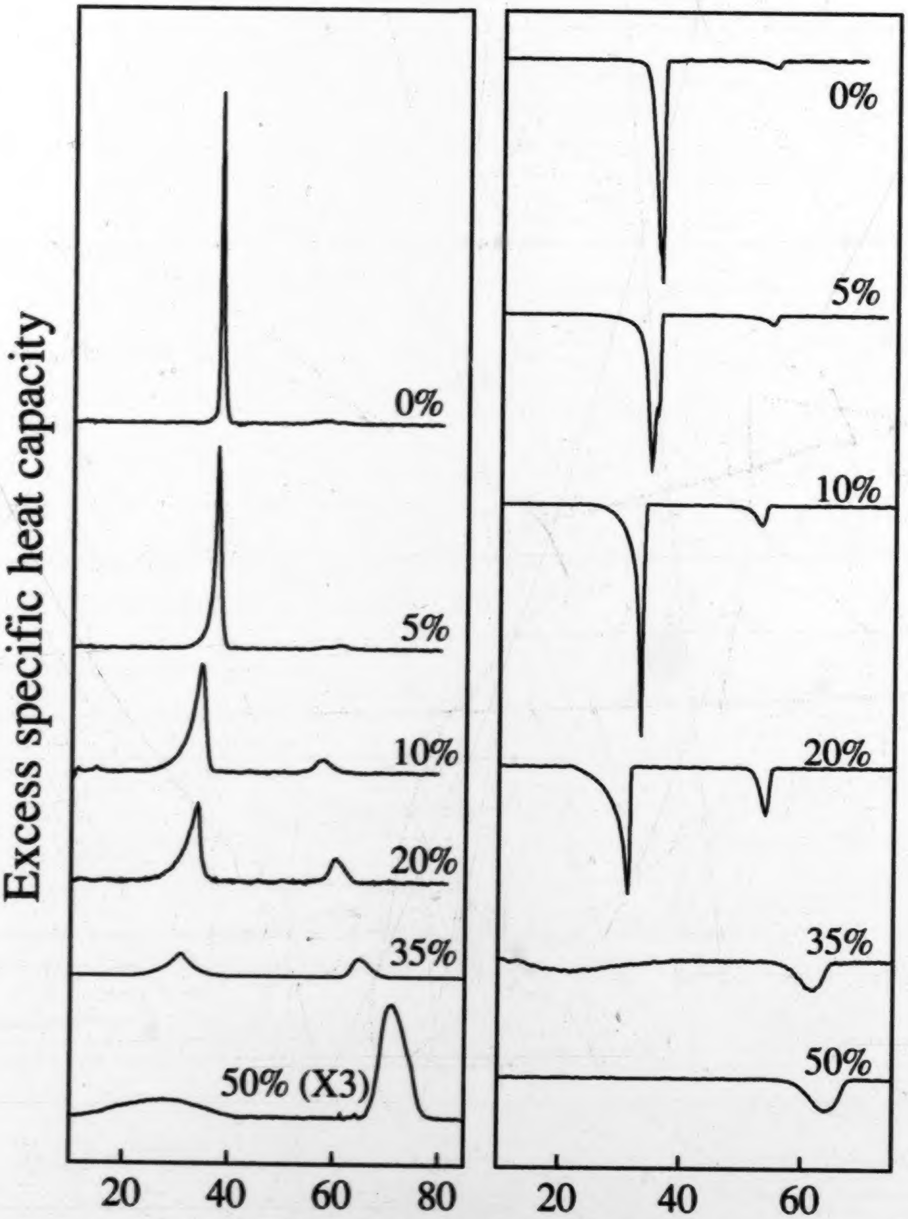

Excess specific heat capacity
0%
5%
10%
20%
35%
50% (X3)
0%
5%
10%
20%
35%
50%
20
40
60
80
20
40
60
Temperature (°C)

Figure IX-6. Representative plots of the temperatures of the phase transition of DSPE (heating ●) (cooling ○) and DEPE (heating ■) (cooling ❑) bilayers as a function of increasing cholesterol levels. In addition, the temperature of the L_α/H_{II} transition (◆) of DEPE bilayers is also shown as a function of increasing cholesterol concentration. Transition temperatures were corrected for differences in heating and cooling scan rates between the various samples analyzed.

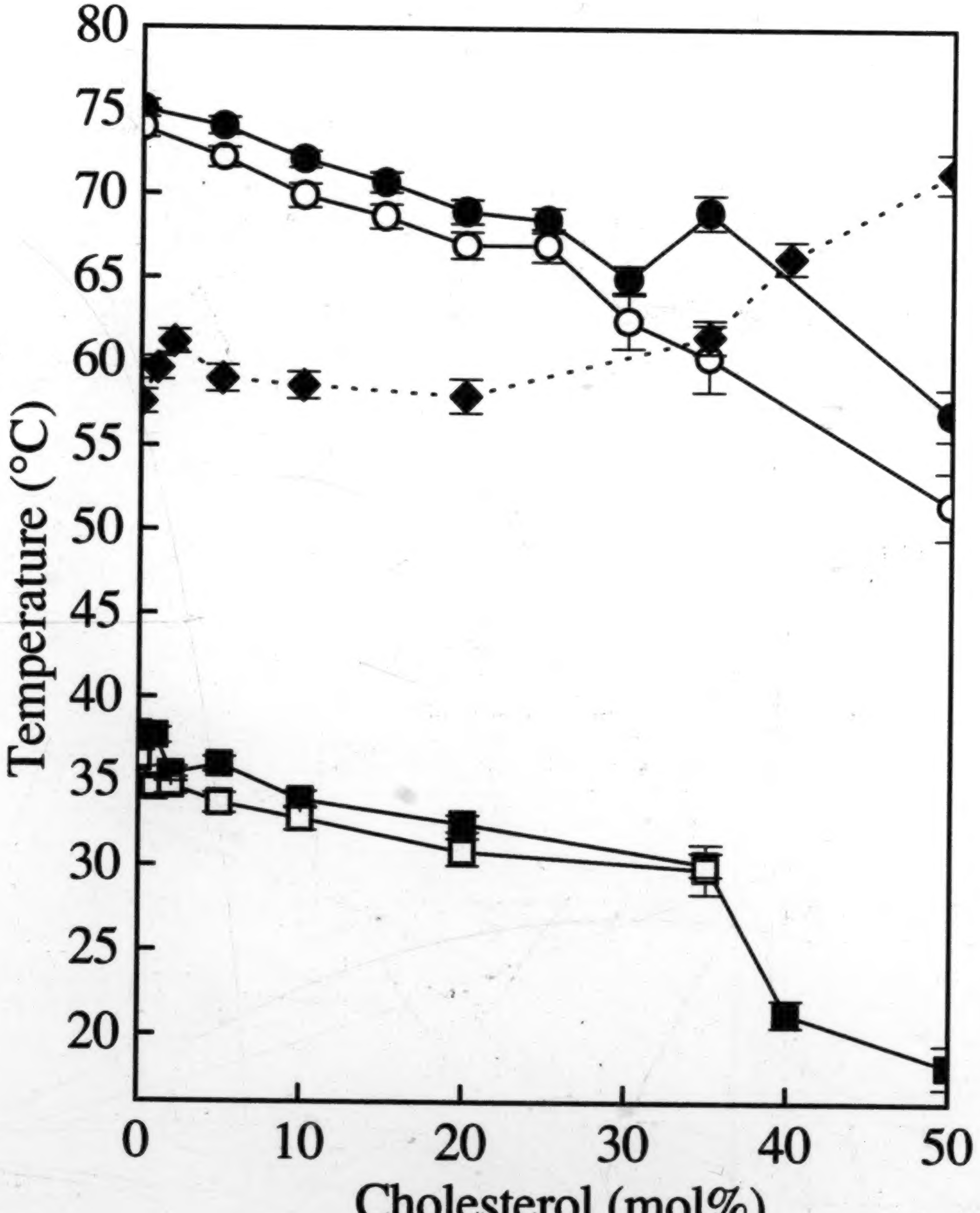
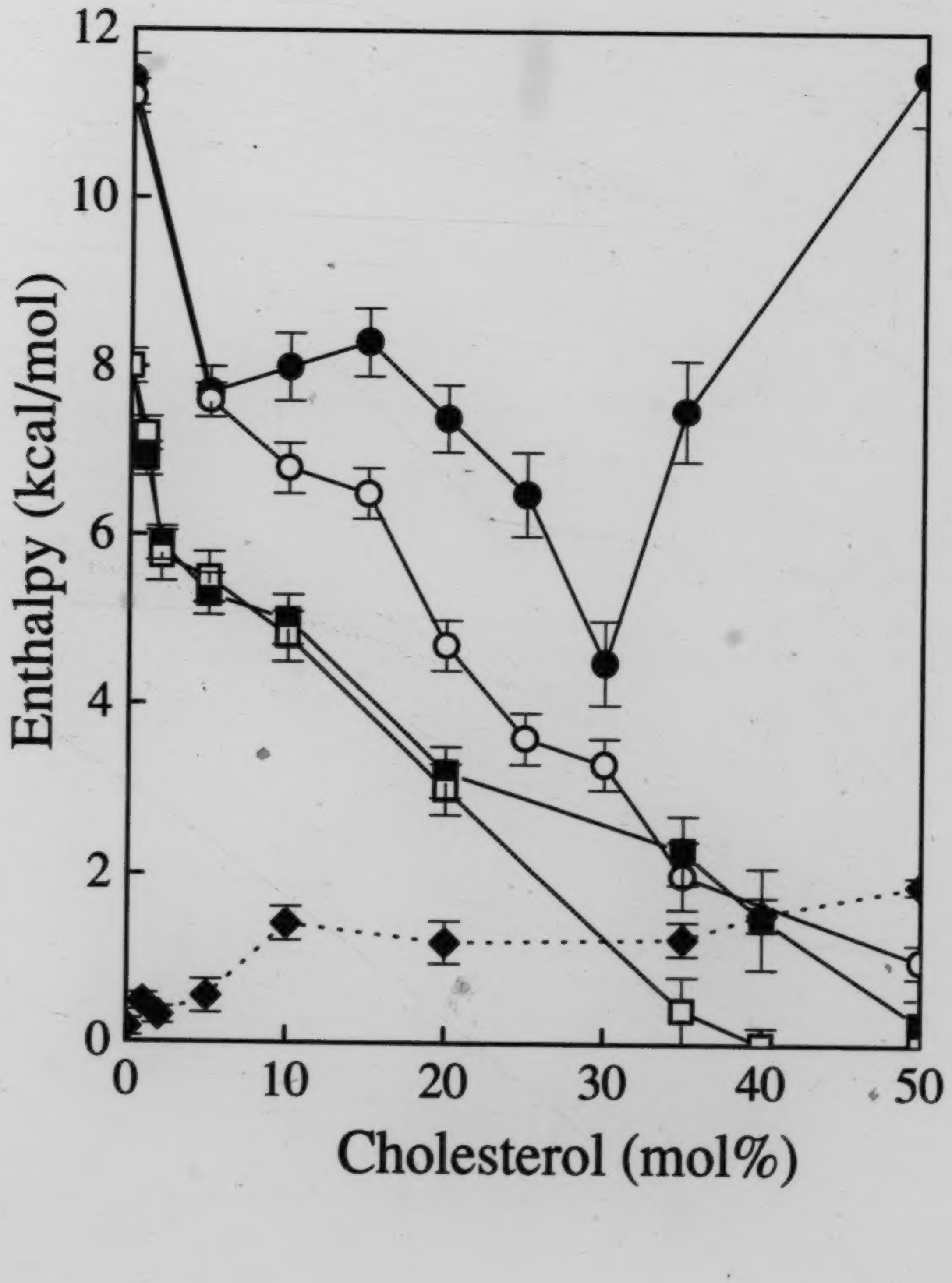

Temperature (°C)
80
75
70
65
60
55
50
45
40
35
30
25
20
0
10
20
30
40
50
Cholesterol (mol%)

Figure IX-7. Representative plots of the enthalpy of the phase transition of DSPE (heating ●) (cooling ○) and DEPE (heating ■) (cooling ❑) bilayers as a function of increasing cholesterol levels. In addition, the enthalpy of the L_α/H_{II} transition (◆) of DEPE bilayers is also shown as a function of increasing cholesterol concentration.



Enthalpy (kcal/mol)
12
10
8
6
4
2
0
0
10
20
30
40
50
Cholesterol (mol%)

Figure IX-8. Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DSPS bilayers containing various concentrations of cholesterol. The scans are adjusted for mass and scan rate.

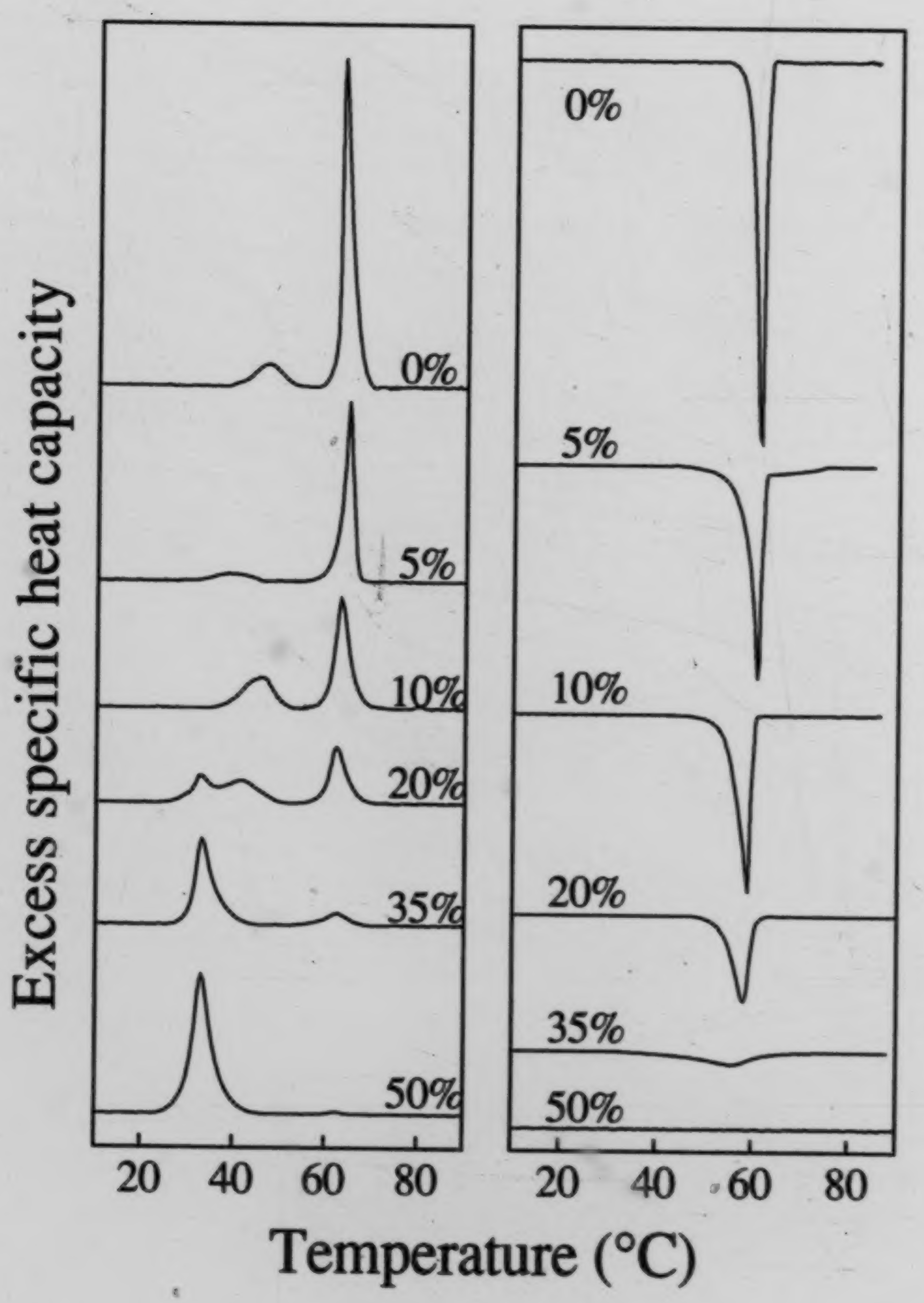

Excess specific heat capacity
0%
5%
10%
20%
35%
50%
0%
5%
10%
20%
35%
50%
20
40
60
80
20
40
60
80
Temperature (°C)

Figure IX-9. Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DEPS bilayers containing various concentrations of cholesterol. The scans are adjusted for mass and scan rate.

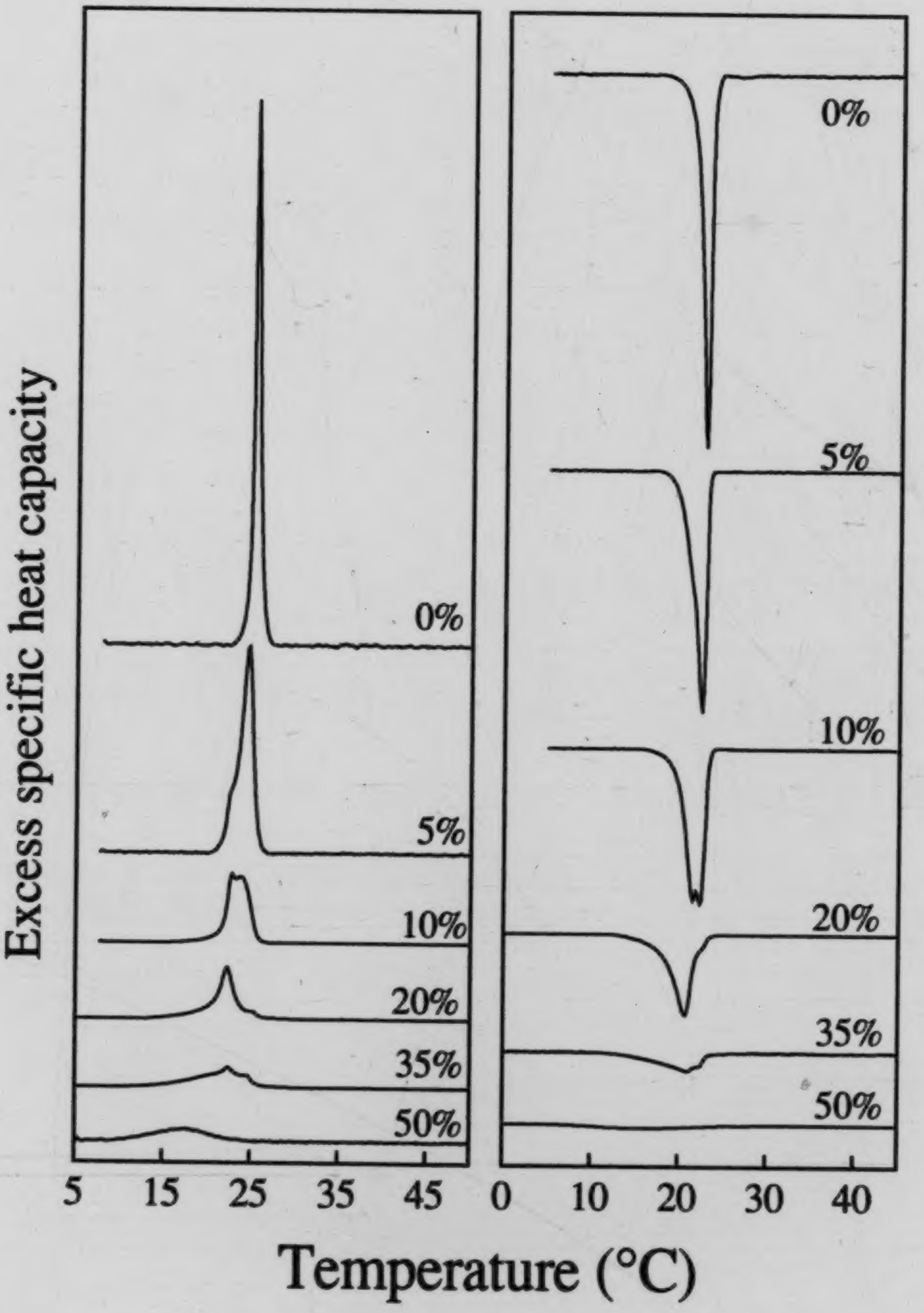

Excess specific heat capacity
0%
5%
10%
20%
35%
50%
5
15
25
35
45
0%
5%
10%
20%
35%
50%
0
10
20
30
40
Temperature (°C)

Figure IX-10. Representative plots of the temperatures of the phase transition of DSPS (heating ●) (cooling ○) and DEPS (heating ■) (cooling □) bilayers as a function of increasing cholesterol level. In addition, the temperature of the L_c/L_β transition (◆) of DSPS bilayers is also shown as a function of increasing cholesterol concentrations. Transition temperatures were corrected for differences in heating and cooling scan rates between the various samples analyzed.

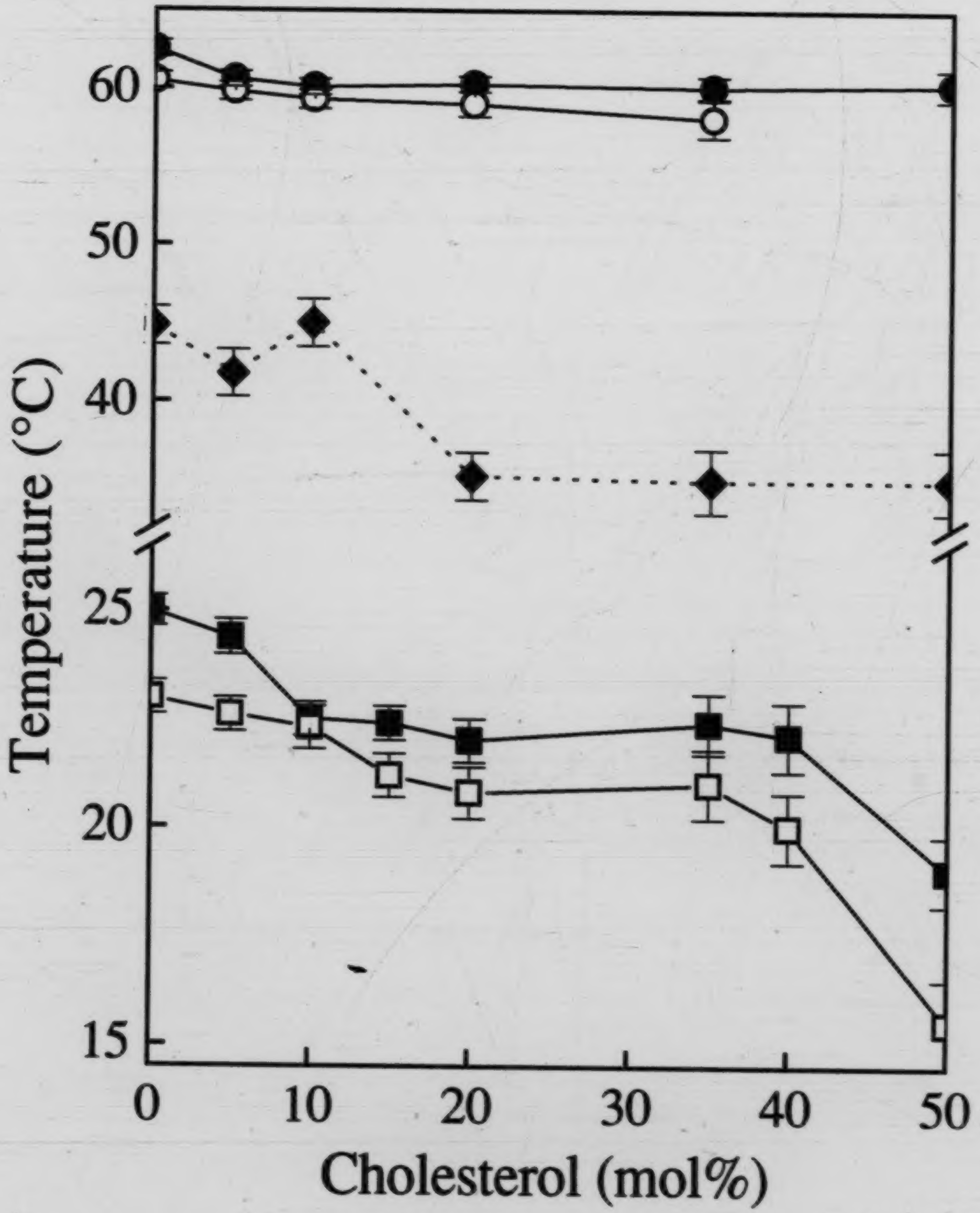
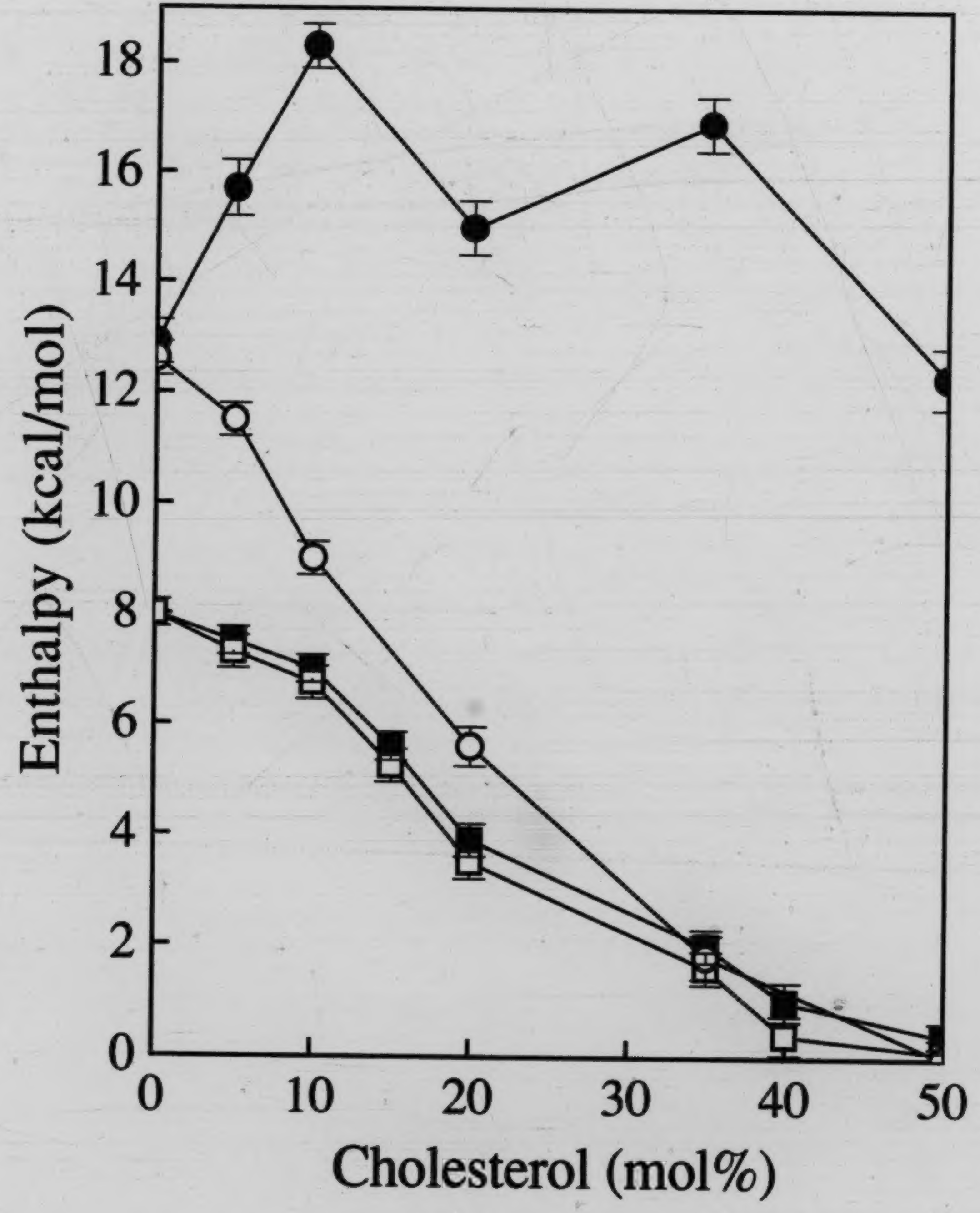

Temperature (°C)
60
50
40
25
20
15
0
10
20
30
40
50
Cholesterol (mol%)

Figure IX-11. Representative plots of the total enthalpy of the phase transitions of DSPS (heating ●) (cooling ○) and DEPS (heating ■) (cooling ❑) bilayers as a function of increasing cholesterol level.


Enthalpy (kcal/mol)
18
16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
Cholesterol (mol%)

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CHAPTER X. DIFFERENTIAL SCANNING CALORIMETRIC STUDY OF THE INTERACTION OF CHOLESTEROL WITH THE MAJOR LIPIDS OF THE *ACHOLEPLASMA LAIDLAWII* B MEMBRANE[10]

INTRODUCTION

Acholeplasma laidlawii B is a member of the mycoplasmas, a phenotypically diverse but monophyletic group of prokaryotic microorganisms that lack a cell wall (see Maniloff, 1992). The mycoplasmas are genetically and morphologically the simplest organisms capable of autonomous replication and they thus provide useful minimal models for the study of a number of problems in molecular and cellular biology. Mycoplasmas are particularly valuable for studies of the structure and function of cell membranes. Being nonphotosynthetic prokaryotes as well as lacking a cell wall or "outer membrane", mycoplasma cells possess only a single limiting membrane. This membrane contains essentially all the cellular lipid and, because these cells are small, a substantial fraction of the total cellular protein as well. Due to the absence of a cell wall, substantial quantities of highly pure membranes can be easily prepared by gentle osmotic lysis followed by differential centrifugation, a practical advantage not offered by other prokaryotic microorganisms (see McElhaney, 1992a).

Another useful property of mycoplasmas in general, and of *A. laidlawii* in particular, is the ability to induce dramatic yet controlled variations in the composition of their membrane lipids. Thus, relatively large quantities of a number of exogenous saturated, unsaturated, branched chain, or alicyclic fatty acids can be biosynthetically incorporated into the membrane phospho- and glycolipids of these organisms. In cases where *de novo* fatty acid biosynthesis is either inhibited or absent, fatty acid-homogeneous membranes (membranes whose lipids contain only a single species of fatty acyl chain) can be produced. Moreover, by growing mycoplasmas in the presence or absence of various quantities of cholesterol or other sterols, the amount of these compounds present in the membrane can be dramatically altered. As well, other manipulations of the growth medium can produce large changes in the types and quantities of the various membrane lipids synthesized by these organisms. The ability to manipulate membrane lipid fatty acid and polar headgroup composition and membrane cholesterol content, and thus to alter the phase state, fluidity and surface properties of the membrane lipid bilayer, makes these organisms ideal for studying the roles of lipids in biological membranes. For these reasons, we probably know more about the structural and functional roles of lipids in the *A. laidlawii* membrane than in any other biological membrane system (see McElhaney, 1992a,b,c).

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[10] A version of this chapter has been published. McMullen, T.P.W., Wong, B.C-.M., Tham, E.L., Lewis, R.N.A.H. and McElhaney, R.N. (1996) *Biochemistry* (in press).

Cholesterol and related sterols are generally found only in eukaryotes, where they play an essential structural and functional role, particularly in the plasma membrane, and they may serve other specific metabolic and regulatory functions as well (see Nes, 1973; Nes and McKean, 1977; Yeagle, 1988; Dahl and Dahl, 1988). However, many mycoplasma species are unique among the prokaryotes in also having an absolute growth requirement for cholesterol or a closely related sterol. Specifically, members of the mycoplasma genera *Anaeroplasma*, *Mycoplasma*, *Spiroplasma* and *Ureaplasma* require exogenous cholesterol for growth and incorporate substantial quantities of this or a closely related sterol into their cell membranes. In contrast, members of the mycoplasma genera *Asteroleplasma* and *Acholeplasma* do not require cholesterol for growth but can incorporate varying but moderate amounts of sterols into their cell membranes. The specificity of the sterol requirement for growth in the former genera, and the mechanism of sterol incorporation and the effects of sterol incorporation of membrane organization and function in both the sterol-requiring and sterol-nonrequiring mycoplasma species, have been extensively studied, particularly in *A. laidlawii* (see McElhaney, 1992a,b,c). Thus it has been demonstrated, using low-sensitivity DSC, that incorporation of moderate amounts of cholesterol into the *A. laidlawii* membrane decreases the temperature, enthalpy and cooperativity of the lipid gel to liquid-crystalline phase transition (de Kruijff *et al.*, 1972, 1973), and that in several *mycoplasma* species the incorporation of large amounts of cholesterol abolishes this transition entirely (Rottem *et al.*, 1973; Rottem, 1981). In general, the effects of cholesterol incorporation on the organization and permeability of various mycoplasma membranes are similar to those observed in model lipid bilayer membranes, where the presence of cholesterol appears to produce a liquid-ordered state, most of whose properties are intermediate between the gel and liquid-crystalline states of the phospholipid bilayer in the absence of cholesterol (see Vist and Davis, 1990; McMullen and McElhaney, 1996).

The sterol-nonrequiring mycoplasmas such as *A. laidlawii* generally contain large quantities of neutral glycolipids, primarily mono- and diglycosyl diacylglycerols, whereas these neutral glycolipids are much reduced or absent in the sterol-requiring mycoplasmas. Efrati *et al.* (1986) have proposed that the lower levels of exogenous cholesterol incorporation characteristic of the sterol-nonrequiring mycoplasmas may be due to their elevated glycolipid content and to a reduced capacity of these glycolipids to incorporate sterols. Moreover, the relatively stronger affinity of cholesterol for phospholipids and its relatively weaker affinity for glycolipids has been invoked to explain the existence of two separate pools of cholesterol which exchange with different kinetics in *A. laidlawii* membrane/liposomal systems (Davis *et al.*, 1984) and which may exist in two different physical states (Monck *et al.*, 1993). In order to investigate these questions further, we studied the effect of the incorporation of increasing quantities of cholesterol on the thermotropic phase behavior of isolated *A. laidlawii* membranes, and of the major phospholipid and of the two major glycolipids thereof, using high-sensitivity DSC. Our results do indeed indicate that the nature of the interaction of cholesterol with each of the major *A. laidlawii* membrane lipids differs considerably. Specifically, we find the apparent miscibility of cholesterol with the neutral glycolipids of this organism to be lower than with the major phospholipid, lending support to the hypothesis of Efrati *et al.* (1986), and to the possibility that the relative immiscibility of cholesterol in the neutral glycolipids may account for the presence of solid-like, laterally segregated, cholesterol-enriched domains in the membranes of this organism (Monck *et al.*, 1993).

MATERIALS AND METHODS

A. laidlawii B cells were grown at 37 °C in a lipid-poor growth medium and harvested at the mid-log phase of growth as described previously (Silvius and McElhaney, 1978a,b; Silvius *et al.*, 1980). Avidin, an inhibitor of *de novo* fatty acid biosynthesis and exogenous fatty acid chain elongation in this organism, was added to the growth medium, as was exogenous elaidic acid. Under these conditions, the fatty acyl groups of the lipids from these "fatty acid-homogeneous membranes" essentially consist only of elaidoyl residues (>98 mol %). The polar lipids were extracted from isolated membranes and the individual lipid components purified, separated, and quantitated as already reported (Monck *et al.*, 1993). The elaidic acid and cholesterol were purchased from NuChek Prep, Inc. (Elysian, MN) and Avanti Polar Lipids (Alabaster, Alabama), respectively, and were used as received.

For the DSC analyses, known amounts of PG, MGDG, DGDG, cholesterol or the *A. laidlawii* total membrane lipids were dissolved in chloroform/methanol (2:1) to make stock solutions from which the lipid/cholesterol mixtures were subsequently made by mixing appropriate amounts of each solution. The binary lipid/cholesterol mixtures were then heated to approximately 30 °C to 50 °C under N_2 to remove the organic solvents and dried under vacuum overnight. The dried lipid/cholesterol mixtures were suspended in a buffer consisting of 50 mM Tris, 10 mM EDTA and 100 mM NaCl at pH 7.4, heated to approximately 10 °C to 20 °C above the phase transition of the mixture, and then vortexed to give a multilamellar suspension. The high-sensitivity DSC thermograms of the lipid/cholesterol suspensions were recorded with a Hart high-sensitivity differential scanning calorimeter (Provo, UT). The heating and cooling scan rates used were progressively increased from 10 to 60 °C/hr as the amount of cholesterol present in the sample increased. In addition, the amount of phospholipid or glycolipid used for HS-DSC analysis was progressively increased from 0.5-2.0 mg for the pure phospholipid or glycolipid to 15 mg for samples containing 50 mol% cholesterol. This protocol has been demonstrated previously to be optimal for accurately detecting the broad, low enthalpy endotherms observed at high cholesterol concentrations (McMullen *et al.*, 1993), particularly in the total membrane lipid and PG samples. The Hart calorimeter was calibrated using solid standards from Hart Scientific as well as aqueous synthetic lipid samples of high purity. Sample runs were repeated at least three times to ensure reproducibility. This protocol provided fully reproducible thermograms. After the HS-DSC scans, the samples were checked for degradation using TLC on Silica Gel G plates with the developing solvent chloroform/methanol/water (70:25:3, by volume) (glycolipids) or chloroform/methanol-/glacial acetic acid/water (60:40:10:4, by volume) (PG) and charred after spraying with sulfuric acid. The plates were overloaded to ensure that small traces of lipid degradation products would be visualized. No degradation was observed.

RESULTS

It is well established from studies of synthetic phospholipid/cholesterol binary mixtures that the detailed nature of the interaction of phospholipids with cholesterol depends on the fatty acyl group chain length and degree of unsaturation as well as on the structure of the phospholipid polar headgroup (see McMullen and McElhaney, 1996). Thus, in order to separate the effects of variations in lipid polar headgroup structure from variations in fatty acid composition, the major membrane lipids from fatty acid-homogeneous *A. laidlawii* membranes were studied. We choose elaidic acid as the exogenous fatty acid with which to grow fatty acid-auxotropic *A. laidlawii* B cells for several reasons. First, this fatty acid produces excellent growth when *de novo* fatty acid biosynthesis and chain elongation are completely inhibited with avidin (Silvius and McElhaney, 1978a,b). Second, the phase transition temperatures of the individual elaidic acid-homogeneous membrane lipids are in a convenient temperature range (5-40 °C) for measurement by high-sensitivity DSC (Silvius *et al.*, 1980; Lewis and McElhaney, 1995). Third, the total membrane lipids are exclusively in the biologically relevant liquid-crystalline state at 37 °C, the optimal growth temperature of this organism (McElhaney, 1974a,b). Fourth, the mean hydrophobic length of elaidic acid-homogeneous membrane lipids is close to that of the cholesterol molecule itself (McMullen *et al.*, 1993), thus maximizing the miscibility of cholesterol with the various *A. laidlawii* membrane phospho- and glycolipids.

The polar headgroup composition of the total membrane lipids from *A. laidlawii* cells grown in the presence of exogenous elaidic acid and avidin, an inhibitor of *de novo* fatty acid biosynthesis and exogenous fatty acid chain elongation in this organism, are shown in Table X-1. The three major lipid components of such elaidic acid-homogeneous membranes are the neutral glycolipids MGDG and DGDG and the anionic phospholipid PG. The glycerylphosphorylated glycolipid GPDGDG is present in much smaller quantities, while the APG and GPMGDG are quite minor lipid components in cells grown under these conditions. In this study we examined the interaction of cholesterol only with the three major lipids of the *A. laidlawii* membrane, since these make up over 90 mol% of the total membrane lipid. PG and DGDG are strongly and weakly lamellar phase-preferring lipids, respectively, under physiologically relevant conditions and exist only as bilayer phases, while MGDG is a strongly reversed phase-preferring lipid, existing in the lamellar phase at lower temperatures and in the H_{II} phase at higher temperatures (see Foht *et al.*, 1995).

Representative high-sensitivity DSC heating and cooling scans of the PG from elaidic acid-homogeneous *A. laidlawii* membranes, alone or in the presence of increasing quantities of cholesterol, are shown in Figure X-1. PG alone exhibits a lower temperature, lower enthalpy pretransition (L_{β}'/P_{β}' transition) near 5 °C and a higher temperature, higher enthalpy main or chain-melting (P_{β}'/L_{α}) phase transition near 11 °C. The pretransition is abolished at very low concentrations of cholesterol (<5 mol%). At low concentrations of cholesterol (<20 mol%), the asymmetric DSC heating endotherms and cooling exotherms of the main transition clearly consist of two components. The phase transition temperature (Figure X-2) and cooperativity of the sharp component decreases slightly with increasing cholesterol incorporation while the transition enthalpy (Figure X-3) decreases markedly, becoming zero by 20 mol% cholesterol. In contrast, the phase transition temperature of the broad component of the DSC endotherms and exotherms increases slightly with increasing cholesterol concentration (Figure X-2) and the cooperativity decreases markedly. The enthalpy of the broad component at first increases as a function of cholesterol incorporation up to a level of about 20 mol% and then decreases to zero at cholesterol concentrations near 50 mol% (Figure X-3). The total enthalpy of the main transition thus decreases more or less linearly with increasing cholesterol incorporation (Figure X-3) and a cooperative chain-melting phase transition is no longer detectable at 50 mol% cholesterol (Figure X-1). The effect of the incorporation of increasing quantities of cholesterol on the thermotropic phase behavior of elaidic acid-enriched PG is essentially identical to that observed for the linear saturated PCs (see McMullen *et al.*, 1993) as well as for DEPC (McMullen *et al.*, 1996a). As previously, we ascribe the sharp and broad components of the DSC heating and cooling thermograms to the melting of the phospholipid hydrocarbon chains of cholesterol-poor and cholesterol-rich PG domains. Note that the effects of cholesterol on the thermotropic phase behavior of the *A. laidlawii* PG are identical in the heating and in the cooling modes, suggesting that cholesterol is fully miscible in both the gel and liquid-crystalline phases of the PG bilayer over the full range of cholesterol concentrations studied, as is also the case for PC's (McMullen *et al.*, 1993, 1996a).

Representative high-sensitivity DSC heating and cooling scans of DGDG from elaidic acid-homogeneous *A. laidlawii* membranes, alone or in the presence of increasing quantities of cholesterol, are presented in Figure X-4. DGDG alone exhibits an asymmetric, moderately cooperative, highly energetic phase transition centered near 33 °C on heating, and a symmetric, highly energetic and highly cooperative phase transition centered near 7 °C upon cooling. Both of these thermotropic events were identified as net interconversions between the L_c and L_α phases by FTIR spectroscopy (unpublished experiments from this laboratory). Although the L_β phase doubtlessly forms transiently upon cooling, the L_β phase of dieladoyl DGDG is unstable with respect to the L_c phase and rapidly converts to the latter on the time scale of the DSC experiment.

In contrast to the results obtained with PG, the DSC thermograms of elaidic acid-enriched cholesterol/DGDG mixtures containing less than 30 mol% are similar to that of pure DGDG bilayers in both the heating and cooling modes, except for a small, low-temperature shoulder on the main transition endotherm observed on heating. Upon heating, the DSC thermograms of cholesterol/DGDG mixtures containing 30 mol% or more cholesterol exhibit at least two major components. From 30 to 50 mol% cholesterol, the observed endothermic peak also broadens significantly, shifts downward in temperature (Figure X-5), and its enthalpy (Figure X-6) decreases sharply as the cholesterol content increases. Nevertheless, even at 50 mol% cholesterol, a broad endothermic event of appreciable enthalpy is still observed at temperatures near 22 °C. The endothermic events observed upon heating of all the DGDG/cholesterol mixtures studied were assigned to L_c/L_α phase transitions by FTIR spectroscopy (data not shown).

Upon cooling, cholesterol/DGDG mixtures containing less than 30 mol% cholesterol exhibit exotherms whose shapes are similar to those of DGDG alone. Moreover, the transition is shifted to slightly lower temperatures and moderate decreases in their enthalpy and cooperativity are observed with increasing cholesterol levels (Figures X-5 and X-6), just as observed in the heating experiments. In this range of cholesterol concentrations, the observed cooling exotherms are still attributable to a L_α/L_c phase transitions as shown by FTIR spectroscopy (data not presented). In the higher range of cholesterol contents, the cooling exotherms also broaden significantly, and their midpoint temperatures and enthalpy values decrease sharply. In these cases, however, these data can be attributed, in part, to changes in the nature of the observed exothermic events as cholesterol content increases. This is because high cholesterol contents progressively decrease the rates at which the L_c phase of DGDG forms from the L_β phase when samples are cooled to temperatures below 7 °C. As a result, a L_α/L_β phase transition becomes detectable and the L_α/L_β and L_β/L_c phase transitions tend to become separated in temperature as cholesterol content increases. Thus, the weak exothermic event exhibited by samples containing 50 mol% cholesterol is primarily a transition between the L_α and L_β phases. However, even at these elevated cholesterol contents, the L_β phase of DGDG is still metastable with respect to the L_c phase of the lipid, and readily converts to the latter when incubated at low temperatures. Thus, it is clear that when cooled to temperatures below 7 °C, DGDG is very prone to form the L_c phase even when mixed with substantial quantities of cholesterol. This result suggests that cholesterol is poorly miscible with, and tends to be excluded from, DGDG bilayers under all conditions where the hydrocarbon chains are frozen. Our results also suggest that cholesterol is not completely miscible even with the liquid-crystalline phase of DGDG, since at lower cholesterol concentrations regions of pure DGDG must exist which can nucleate L_c phase formation on cooling.

Representative high-sensitivity DSC heating and cooling scans of MGDG from elaidic acid-homogeneous *A. laidlawii* membranes, alone or in the presence of increasing quantities of cholesterol, are shown in Figure X-7. As observed also for the DGDG, the MGDG alone forms an L_c phase when exposed to low temperatures, and upon heating the L_c phase converts directly to the H_{II} phase near 39 °C. (confirmed by unpublished X-ray and FTIR data from this laboratory). At cholesterol concentrations up to 30 mol%, the relatively sharp, highly enthalpic L_c/H_{II} phase transition endotherm is little affected by the presence of cholesterol, except for a small decrease in transition temperature (Figure X-8). At cholesterol concentrations exceeding 30 mol% a broad, second endothermic process is detectable but the overall transition enthalpy does not change significantly (Figure X-9). However, at 50 mol% cholesterol, the L_c chain-melting endotherm is completely abolished and only a new endotherm centered near 50 °C persists. As shown below, the new endotherm appearing near 50 °C is probably attributable to a L_α/H_{II} phase transition. Thus cholesterol at low concentration appears to be only sparingly miscible with MGDG at low temperatures, where the L_c phase predominates, but at much higher concentrations cholesterol can apparently be solubilized in MGDG bilayers and can inhibit both L_β and L_c phase formation.

Upon cooling, the *A. laidlawii* MGDG alone exhibits two exotherms, a higher temperature, lower enthalpy H_{II}/L_α near 31 °C and a lower temperature, higher enthalpy L_α/L_β phase transition centered near 28 °C. In this case, however, the presence of cholesterol at all concentrations produces a significant upward shift in the H_{II}/L_α, a small downward shift in the L_α/L_β phase transition temperature (Figure X-8), and a small decrease in the cooperativity of both transitions. At higher cholesterol concentration, the

H_{II}/L_{α} phase transition becomes more strongly shifted to higher temperatures (Figure X-8) and becomes broader but actually increases in enthalpy (Figure X-9). In contrast, the L_{α}/L_{β} phase transition exotherm is shifted strongly to lower temperatures (Figure X-8) and is markedly reduced in enthalpy (Figure X-9). These results suggest that cholesterol has a much greater miscibility with the L_{β} and H_{II} states than with the L_c state of elaidic acid-enriched MGDG aqueous dispersions. Moreover, cholesterol incorporation into the MGDG L_{α} phase bilayer inhibits the formation of the L_c and L_{β} phases which would normally form at lower temperatures. Thus, the cholesterol which is incorporated stabilizes the L_{α} phase of aqueous dispersions of MGDG relative to both the L_{β} and H_{II} phases, thus markedly increasing the temperature range over which the L_{α} phase is stable upon both heating and cooling.

Representative high-sensitivity DSC heating and cooling scans of the total membrane lipids from elaidic acid-homogeneous *A. laidlawii* cells, in the presence or absence of various amounts of cholesterol, are presented in Figure X-10. In the absence of added cholesterol, the total membrane lipids exhibit a fairly broad, ramplike gel to liquid-crystalline phase temperature with an overall midpoint temperature near 22 °C, but consisting of at least two major components with transition temperatures near 19 °C and 24 °C. Note that the width of the chain-melting phase transition of the total membrane lipids is much greater than that of the individual major membrane lipids, presumably due only to the membrane lipid polar headgroup compositional heterogeneity, since there is no heterogeneity in the fatty acid composition of these membrane lipids. Note also that the phase transition midpoint temperature of the total membrane lipids is roughly intermediate between the L_{β}/L_{α} phase transition temperatures of the lower-melting PG (11 °C) and DGDG (13.4 °C), and of the higher-melting MGDG (27.9 °C), components. Taken together, these observations indicate that the individual lipid components of the *A. laidlawii* membrane have an appreciable mutual miscibility but that mixing is not ideal.

As the exogenous cholesterol concentration is increased from 0 to 20 mol%, the overall phase transition midpoint temperature of the total membrane lipids is shifted downward (Figure X-11) but the two components of this transition remain and the enthalpy of the phase transition is reduced (Figure X-12). From 30 to 50 mol% cholesterol, the phase transition continues to decrease in temperature and enthalpy but the transition becomes single component. Moreover, some residual enthalpy remains at 50 mol% cholesterol. Interestingly, the width of the already broad gel to liquid-crystalline phase transition of the total membrane lipids does not appear to increase appreciably as the concentration of cholesterol added increases, at least up to 30 mol% cholesterol. This result is quite different than is observed for the PG component of elaidic acid-homogeneous *A. laidlawii* membranes or for synthetic PC's (see McMullen *et al.*, 1993, 1996a), where a distinct broadening of the chain-melting phase transition is noted even at quite low cholesterol concentrations and where the broadening increases smoothly and progressively with increases in cholesterol incorporation, the phase transition being abolished at 50 mol% cholesterol. However, the *A. laidlawii* neutral glycolipids also exhibit only small increases in the width of their gel to liquid/crystalline phase transitions over the cholesterol concentration range 0 to 30 mol% and a residual enthalpy at 50 mol% cholesterol, as do the total membrane lipids. It is also possible that cholesterol could increase the mutual solubility of the *A. laidlawii* membrane lipids, particularly at high concentrations, as has been demonstrated in some but not all laterally phase separated ternary cholesterol/phospholipid or cholesterol/glycolipid systems (Silvius, 1992). Such an effect may counteract the intrinsic broadening of the chain-melting phase transition of the individual membrane lipids by cholesterol which is observed especially at high cholesterol levels.

DISCUSSION

We have shown previously that the relative miscibility of cholesterol (McMullen *et al.*, 1993, 1996a,b) and various side chain-truncated cholesterol analogues (McMullen *et al.*, 1995; Vilchèze *et al.*, 1996) with the gel and liquid-crystalline phases of PC and PE bilayers can be determined by the effect of the incorporation of various concentrations of these sterols on the enthalpy of the gel to liquid-crystalline phase transition as monitored by DSC. In binary cholesterol/phospholipid systems exhibiting complete miscibility in both the solid and fluid states, the transition enthalpy decreases smoothly to zero as the sterol content of the mixtures increases from 0 to 50 mol%, in both the heating and cooling modes. Similarly, in binary systems exhibiting only gel-state sterol immiscibility, the enthalpy of the gel to liquid-crystalline phase transition measured in the heating mode first declines (and may reach a plateau) as cholesterol content increases but that in all cases the chain-melting phase transition is not abolished at 50 mol% cholesterol; however, when measured in the cooling mode, the transition enthalpy declines smoothly to zero at 50 mol% cholesterol. Finally, if cholesterol shows limited solubility in both the gel and liquid-crystalline phases of a particular lipid, then a phase transition will remain detectable at 50 mol % sterol in both the heating and cooling modes, with the magnitude of this residual enthalpy providing an approximate measure of the degree of sterol immiscibility in each phase.

It is clear from our DSC results that the miscibility of cholesterol in the gel and liquid-crystalline lamellar phases of the three major lipids of the *A. laidlawii* membrane differs markedly. Thus elaidic acid-enriched cholesterol/PG mixtures exhibit complete miscibility in both the gel and liquid-crystalline phases, while the cholesterol/DGDG and cholesterol/MGDG mixtures exhibit significant immiscibility, particularly in the gel state and particularly at lower cholesterol concentrations. The reason for the relative immiscibility of cholesterol in the neutral glycolipid bilayers generally is probably due to the relatively tightly packed, extensively hydrogen-bonding bilayers formed by these glycolipids, particularly in the gel state, which tends to exclude cholesterol into separate, laterally segregated domains. The reason for the increased miscibility of cholesterol with MGDG and DGDG bilayers at higher cholesterol concentrations or at higher temperatures may reflect a disruption of glycolipid-glycolipid hydrogen bonding and other interactions, thus loosening the bilayer and permitting a more facile penetration of cholesterol into the glycolipid matrix. In this regard it is interesting to note that we have shown that cholesterol is fully miscible in the gel and liquid-crystalline states of synthetic saturated, mixed-chain and *trans*-unsaturated PC bilayers (McMullen *et al.*, 1993, 1995; Vilchèze, 1996), while in saturated (McMullen *et al.*, 1996b) or *trans*-unsaturated PE bilayers (McMullen *et al.*, 1996a) cholesterol exhibits gel phase immiscibility. Again, calorimetric and spectroscopic data clearly indicate that PE forms higher melting, more densely packed bilayers, particularly in the gel state, as compared to PC, presumably because of the stronger electrostatic and hydrogen-bonding interactions in the polar headgroup region of the former phospholipid (see Lewis *et al.*, 1993 and references therein). Thus the *A. laidlawii* PG behaves like synthetic and natural PC's in its interaction with cholesterol while the MGDG and DGDG behave more like PE, except that these neutral glycolipids exhibit some immiscibility with cholesterol also in the liquid-crystalline state, whereas PE does not (McMullen *et al.*, 1996a). The relative immiscibility of cholesterol on the total membrane lipids of elaidic acid-homogeneous *A. laidlawii* cells is doubtlessly due to the presence of substantial quantities of neutral glycolipids in the cell membrane of this organism (see Table X-1).

The difference in the nature of the interaction of cholesterol with PG, and with MGDG and DGDG, is also manifested in the direction and magnitude in the shift in the gel to liquid-crystalline phase transition temperature induced by presence of this sterol. In the case of PG, the L_β/L_α phase transition temperature is increased slightly by the addition of cholesterol, but the upward shift is modest, being only about 3 °C as one approaches 50 mol% cholesterol. However, for the MGDG and DGDG, a substantial decrease in the L_β/L_α phase transition temperatures is observed. In particular this reduction in the phase transition temperature is about 16 °C and 6 °C, respectively, at 50 mol% cholesterol. Interestingly, a similar slight upward shift in the L_β/L_α phase transition is noted upon the addition of cholesterol to synthetic PCs (McMullen *et al.*, 1993, 1995, 1996a) and a similar major downward shift in the L_β/L_α phase transition temperature is noted in synthetic PEs (McMullen *et al.*, 1996b). Thus, it appears that in lipids forming low-melting gel state bilayers characterized by relatively weak lipid polar headgroup electrostatic and hydrogen bonding interactions, cholesterol stabilizes the gel state relative to the liquid-crystalline state, probably by relieving the crowding of the relatively large polar headgroups (in the case of PC), by decreasing the electrostatic repulsion of like charges (in the case of PG), and by providing additional attractive hydrogen-bonding interactions at the phospholipid bilayer polar/apolar interface (both phospholipids) (see McMullen and McElhaney, 1996). In contrast, the effect of cholesterol incorporation on the higher-melting lipids is to decrease the stability of the gel or crystalline relative to the liquid-crystalline state, presumably by increasing the spacing of the lipid polar headgroups and thus disrupting their initially relatively strong electrostatic and/or hydrogen bonding attractive interactions. In this regard, note that the reduction in the phase transition temperature of the higher melting MGDG component is considerably greater than that of the lower melting DGDG component of identical fatty acid composition, as predicted from their relative phase transition temperatures.

Efrati *et al.* (1986) have proposed that the difference in the exogenous cholesterol incorporation capacities of *Acholeplasma* and *Mycoplasma* species may be due to the much higher membrane neutral glycolipid contents of the former and to a reduced capacity of glycolipids to solubilize cholesterol. This proposal was supported by a statistical analysis of a large number of *Acholeplasma* species and strains which revealed a significant negative correlation between the glycolipid content of the cell membrane and the level of incorporation of cholesterol by growing cells. Also given as evidence for this hypothesis was the observation that maximum cholesterol uptake increases in aging cells in conjunction with decreased neutral glycolipid levels. Moreover, Efrati *et al.* (1981) had shown earlier that the degradation of 90-94% of the PG and DPG from the *A. laidlawii* membrane resulted in a disproportionate decrease in cholesterol uptake. Specifically, although these two phospholipids made up only 30 wt% of the total membrane lipids, their hydrolysis by phospholipase A_2 (and the prior removal of the hydrolysis products from the membrane) resulted in a decrease in cholesterol incorporation of 55%, suggesting that the neutral glycolipids remaining in the membranes must have a reduced capacity for cholesterol uptake. The results of our present DSC study of the interactions of cholesterol with the three major lipids of the *A. laidlawii* membrane would seem to support the hypothesis of Efrati *et al.* (1986) in that the maximum amount of cholesterol which can be incorporated into PG bilayers seems greater than that which can be incorporated into DGDG and MGDG bilayers. In this regard it is noteworthy that the glycosphingolipids from eukaryotic cells generally show a reduced ability to solubilize cholesterol as compared to most phospholipids, and that the ability of glycosphingolipids to solubilize cholesterol also depends more markedly on the exogenous cholesterol concentration than is the case for most phospholipids (McCabe and Green, 1977). However, Efrati *et al.* (1981) stated, without presenting data, that the neutral glycolipid fraction from *A. laidlawii* membrane can solubilize the same maximum amount of cholesterol as can the phospholipid or phosphoglycolipid fractions when cosonicated, a surprising observation in view of the present results and those of others discussed above.

The existence of two pools of cholesterol in the *A. laidlawii* membrane has also been suggested to support the hypothesis of Efrati *et al.* (1986). Thus Davis *et al.* (1984) reported that in intact cells, and in isolated membranes, about half of the cholesterol associated with the membrane exchanges relatively rapidly with egg PC/cholesterol vesicles while the other half exchanges much more slowly. They suggested that the rapidly exchanging cholesterol pool could result from cholesterol binding weakly to the membrane glycolipids and the slowly exchanging pool to cholesterol bound strongly to the membrane phospholipids. However, the removal of most of the phospholipid by phospholipase A_2 hydrolysis resulted in a single kinetic pool of cholesterol which exchanged at a rate only slightly faster than the rate of the slowly exchanging cholesterol pool in untreated cells, which is not the result predicted if the rapidly exchanging pool in untreated cells is due to glycolipid-bound cholesterol. In this regard, we have indeed confirmed the existence of two structurally different pools of cholesterol in *A. laidlawii* membranes (Monck *et al.*, 1993), one solubilized in the lipid bilayer and another in a solid-like form whose location in the membrane could not be determined, and we have also shown that heating or lyophilization and rehydration at high temperature converts the solid-like form of cholesterol into bilayer-solubilized cholesterol. In principle, this conversion of the solid-like cholesterol pool into a lipid bilayer-dispersed form could be due to an increased solubilization of free cholesterol by the membrane neutral glycolipids induced by these treatments. However, whether or not the existence of the solid-like cholesterol pool is related to the presence of the neutral glycolipids in the membrane of this organism remains to be determined.

It is instructive to compare the effects of cholesterol on the L_α/H_{II} phase transition of synthetic DEPE and the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes. The L_α/H_{II} phase transition temperature of DEPE has been shown to decrease moderately from 0 to 10 mol% cholesterol, to plateau at 10 to 30 mol% cholesterol, and then to increase markedly at higher cholesterol concentrations, while the phase transition enthalpy increases markedly (over 3-fold) and apparently linearly over this same sterol concentration range. Moreover, the DEPE L_α/H_{II} phase transition DSC endotherm is clearly two-component over this entire range of cholesterol concentrations (Epand and Bottega, 1987; Cheetham *et al.* 1989). In contrast, the L_α/H_{II} phase transition temperature of MGDG increases regularly and supralinearly over the cholesterol concentration range 0 to 50 mol% and the transition enthalpy increases by less than 2-fold from 0 to 20 mol%, where the enthalpy values plateau. Moreover, the MGDG DSC exotherms are not obviously two-component except at the highest cholesterol concentrations. Thus, just as the effect of cholesterol on the L_α/H_{II} phase transition of synthetic and natural PEs depends to a considerable extent on the length and degree of unsaturation of the fatty acyl chains (Dekker *et al.*, 1983; Epand and Bottega, 1987; Cheetham *et al.*, 1989), so too does it depend on the polar headgroup structure of the lipid when the composition of the fatty acyl chains are held constant.

The effect of any additive on the L_α/H_{II} phase transition temperature of a nonlamellar phase-forming lipid is usually explained on the basis of the effective molecular shape of that additive and by its effect on the dynamic shape of the host lipid molecules with which it interacts (see Lewis *et al.*, 1996). Because of the small size and cross-sectional area of its polar headgroup in comparison to the relatively large size and cross-sectional area of its nonpolar fused ring system, the cholesterol molecule is usually considered to have an inverted cone shape and should therefore promote H_{II} phase formation. However, cholesterol also orders the hydrocarbon chains of the host lipid bilayer and increases its hydrophobic thickness, factors which should reduce the propensity of the host lipid to form a reversed phase, since this phase is characterized by more highly disordered hydrocarbon chains and by monolayers of decreased thickness. Moreover, the incorporation of cholesterol will increase the spacing of the polar headgroups in the bilayer, which for the small, strongly interacting polar headgroups of PE and MGDG molecules, should decrease the intrinsic radius of curvature of each monolayer and thus the propensity to form the H_{II} phase. Similarly, the reduction of the L_β/L_α phase transition temperature noted with both synthetic and natural PEs and MGDG can be explained by the effect of cholesterol in disordering the hydrocarbon chains of the host lipid bilayer at low temperatures, which also thus increases the hydrophobic thickness of the lipid bilayer and reduces the magnitude of the attractive interactions between the polar headgroups of adjacent phospho- or glycolipid molecules, thus promoting L_α over L_β phase formation. However, additional work will be required to explain why the balance of forces always favors H_{II} phase formation in elaidic acid-enriched MGDG but may favor either L_α or H_{II} phase formation in DEPE, depending on the concentration of cholesterol.

Figure X-1. Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the PG from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol.

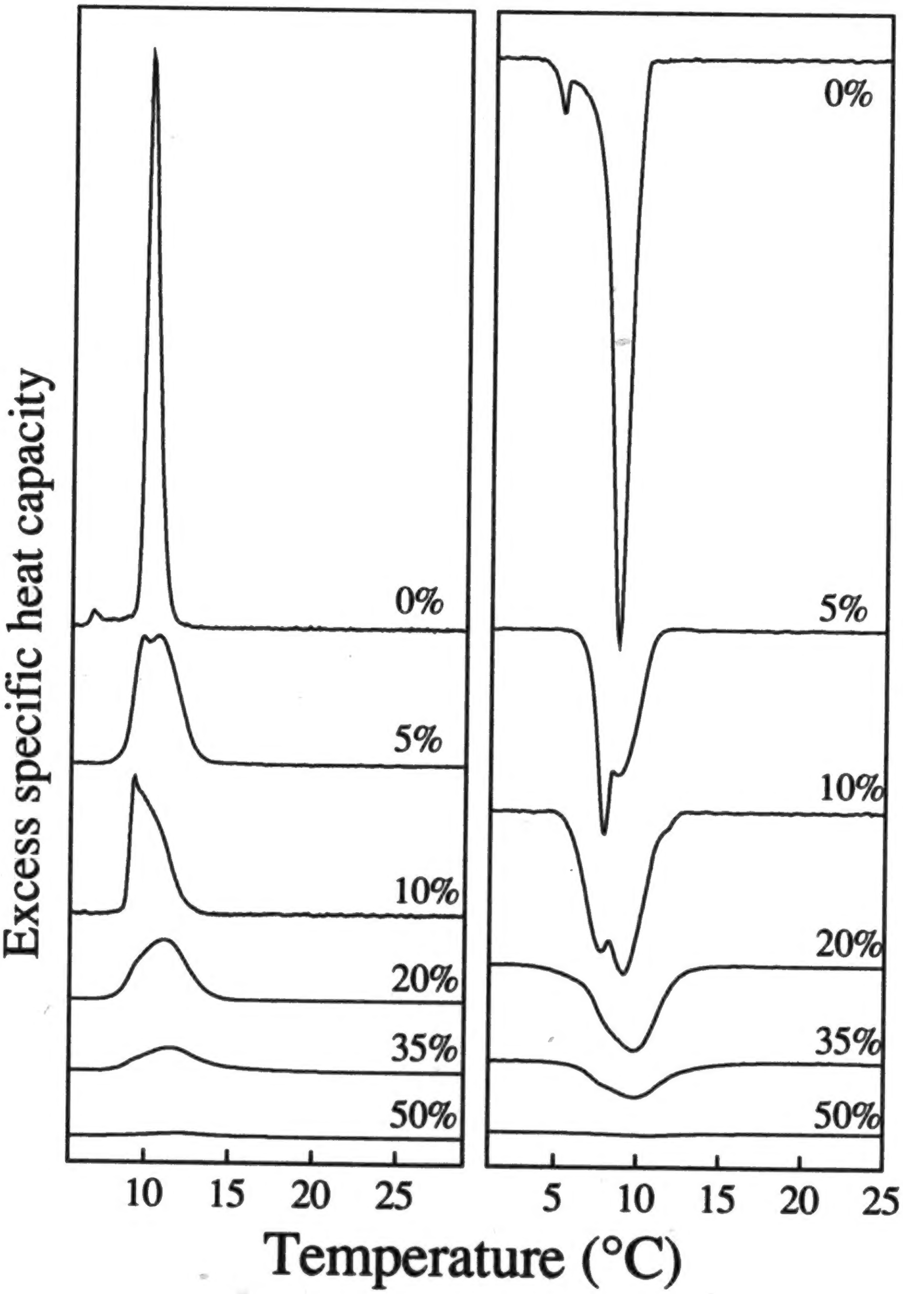
Excess specific heat capacity
0%
5%
10%
20%
35%
50%
10
15
20
25
0%
5%
10%
20%
35%
50%
5
10
15
20
25
Temperature (°C)

Figure X-2. Representative plots of the temperatures of the broad (heating ▼) (cooling ▽) and sharp (heating ▲) (cooling Δ) components of the main phase transition of aqueous dispersions of the PG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration, taken here from the heating scans. Transition temperatures were corrected for differences in heating scan rates between the various samples analyzed.

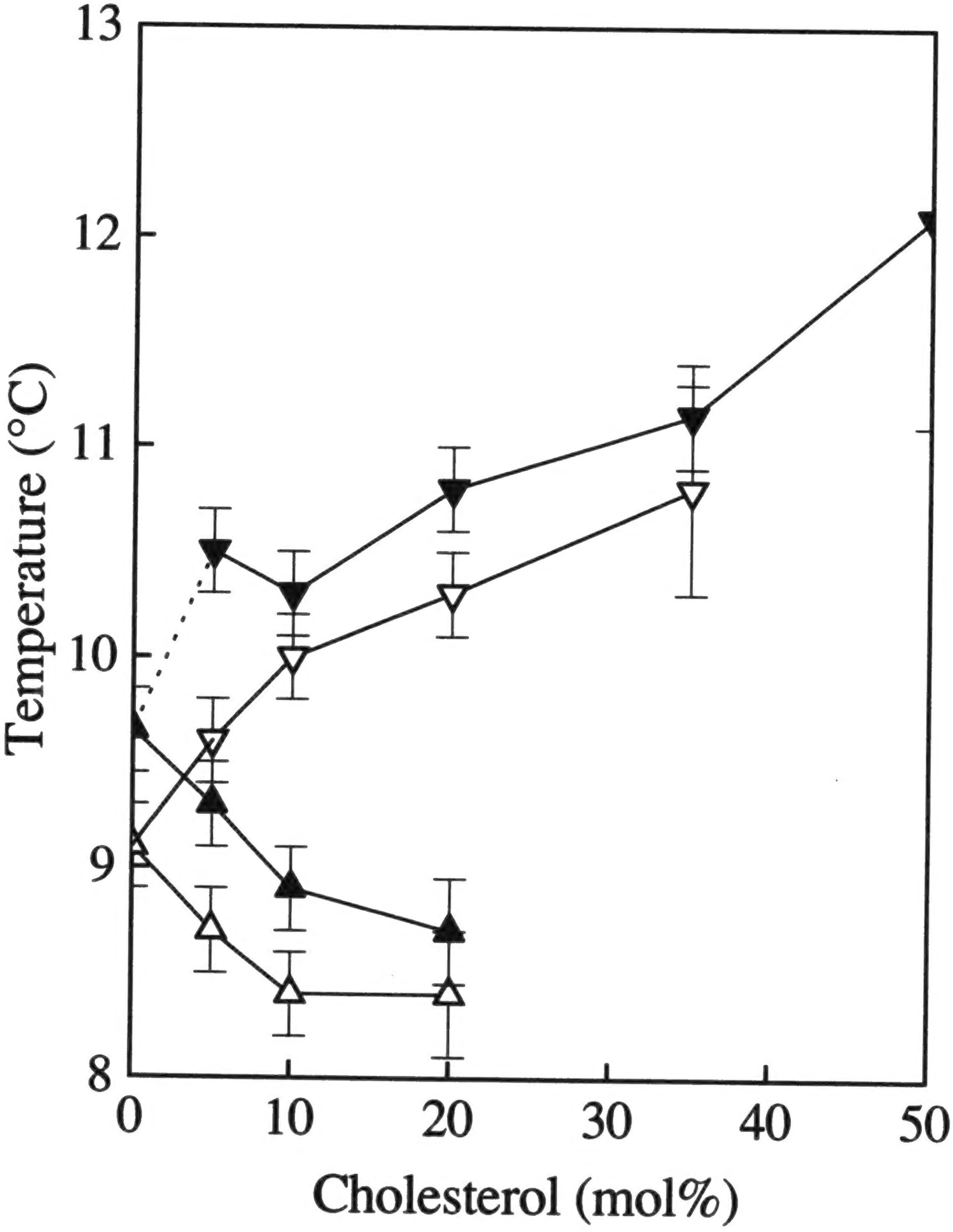
13
12
11
10
9
8
Temperature (°C)
0
10
20
30
40
50
Cholesterol (mol%)

Figure X-3. Representative plots of the total enthalpy (heating ●) (cooling ❍) and the enthalpy of the sharp (▲) broad (▼) components, of the main phase transition of aqueous dispersions of the PG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

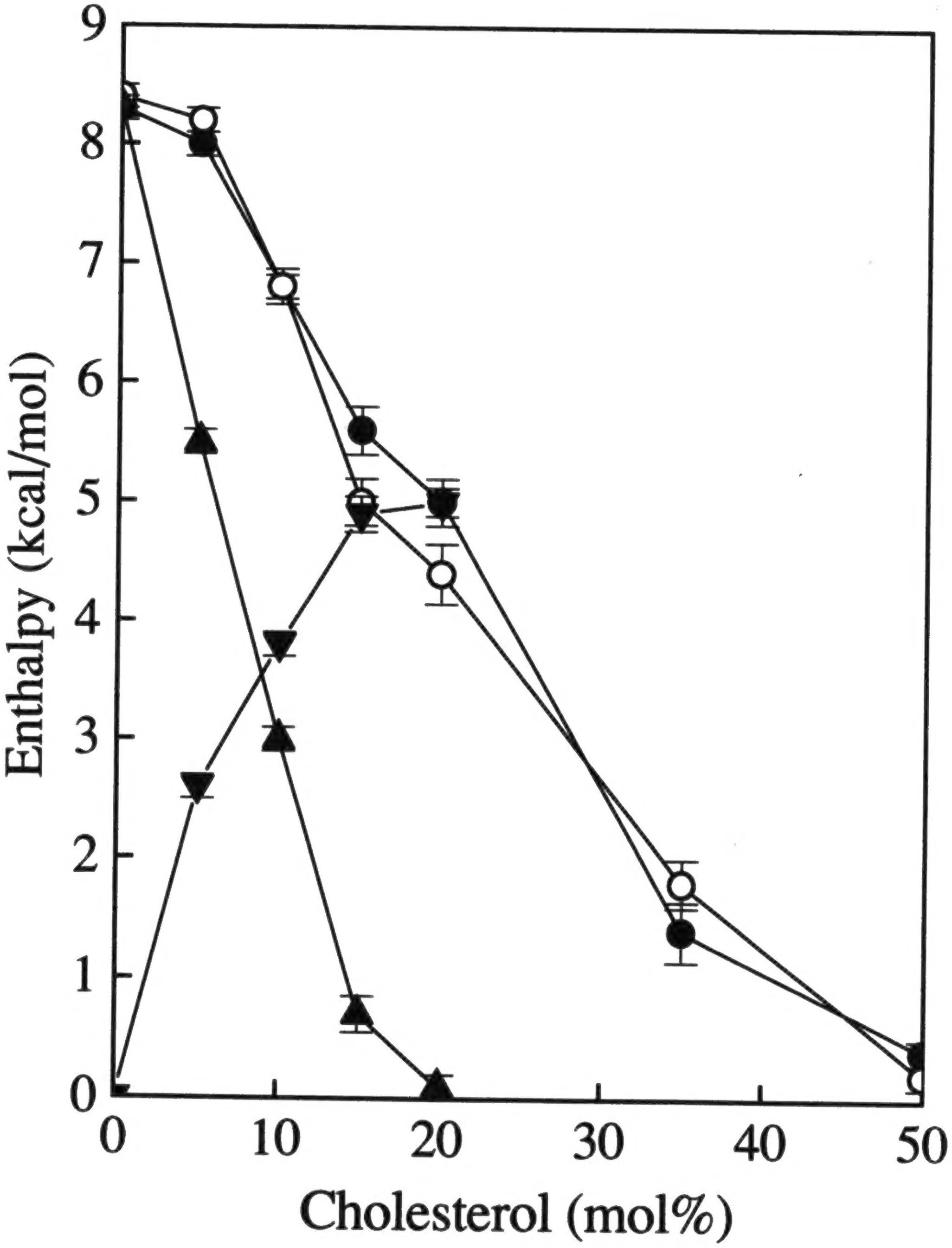
Enthalpy (kcal/mol)
Cholesterol (mol%)
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50

Figure X-4. Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the DGDG from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol.

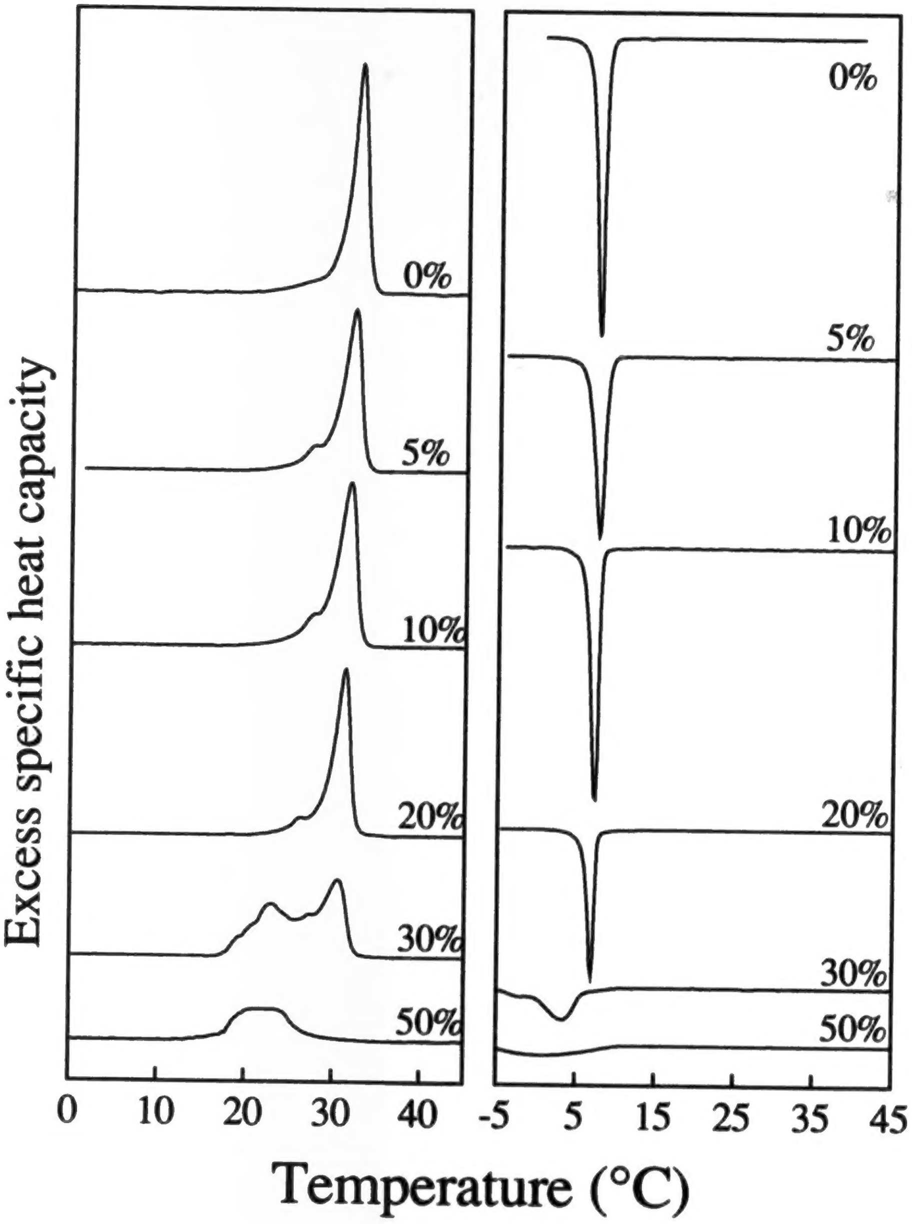
Excess specific heat capacity
0%
5%
10%
20%
30%
50%
0
10
20
30
40
0%
5%
10%
20%
30%
50%
-5
5
15
25
35
45
Temperature (°C)

Figure X-5. Representative plots of the temperatures of the L_c/L_α (heating ●) and L_α/L_β (cooling ○) phase transitions of aqueous dispersions of DGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. Transition temperatures were corrected for differences in heating and cooling scan rates between the various samples analyzed.

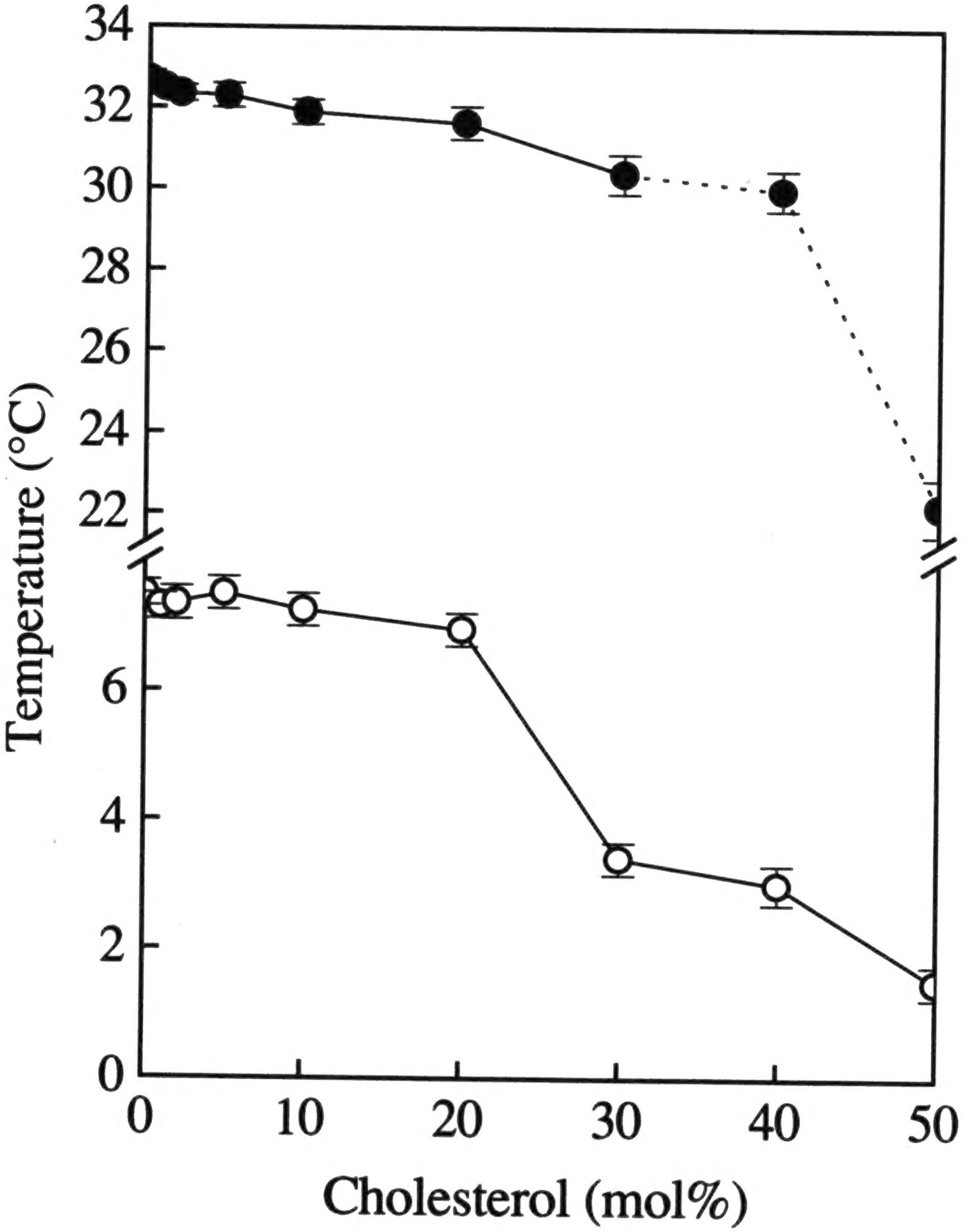
34
32
30
28
26
24
22
6
4
2
0
Temperature (°C)
0
10
20
30
40
50
Cholesterol (mol%)

Figure X-6. Representative plots of the total enthalpy of the L_c/L_α (heating ●) and the L_α/L_β (cooling ○) phase transitions of aqueous dispersions of the DGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

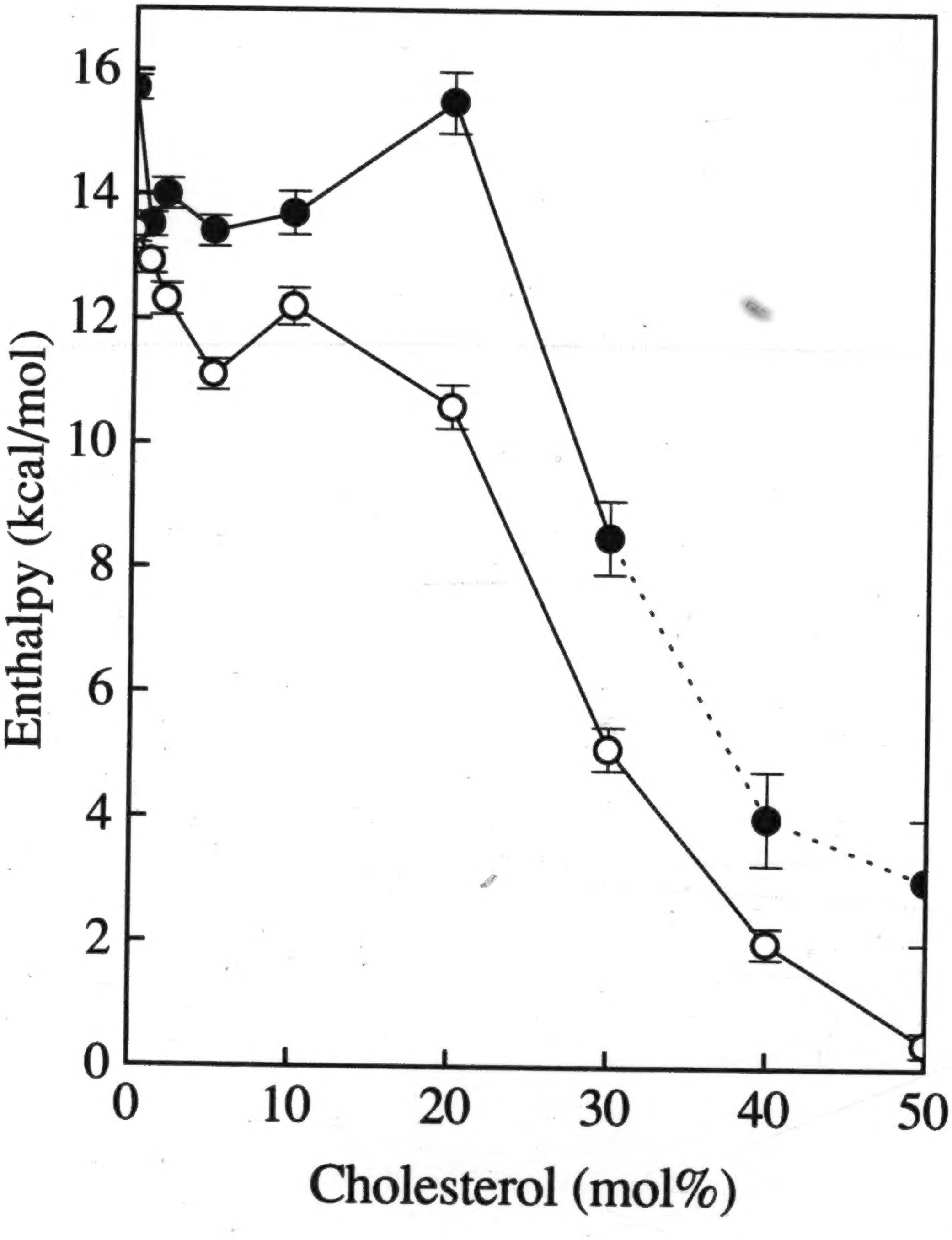
16
14
12
10
8
6
4
2
0
Enthalpy (kcal/mol)
0
10
20
30
40
50
Cholesterol (mol%)

Figure X-7. Representative DSC heating (left panel) and cooling (right panel) scans of aqueous dispersions of the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol.

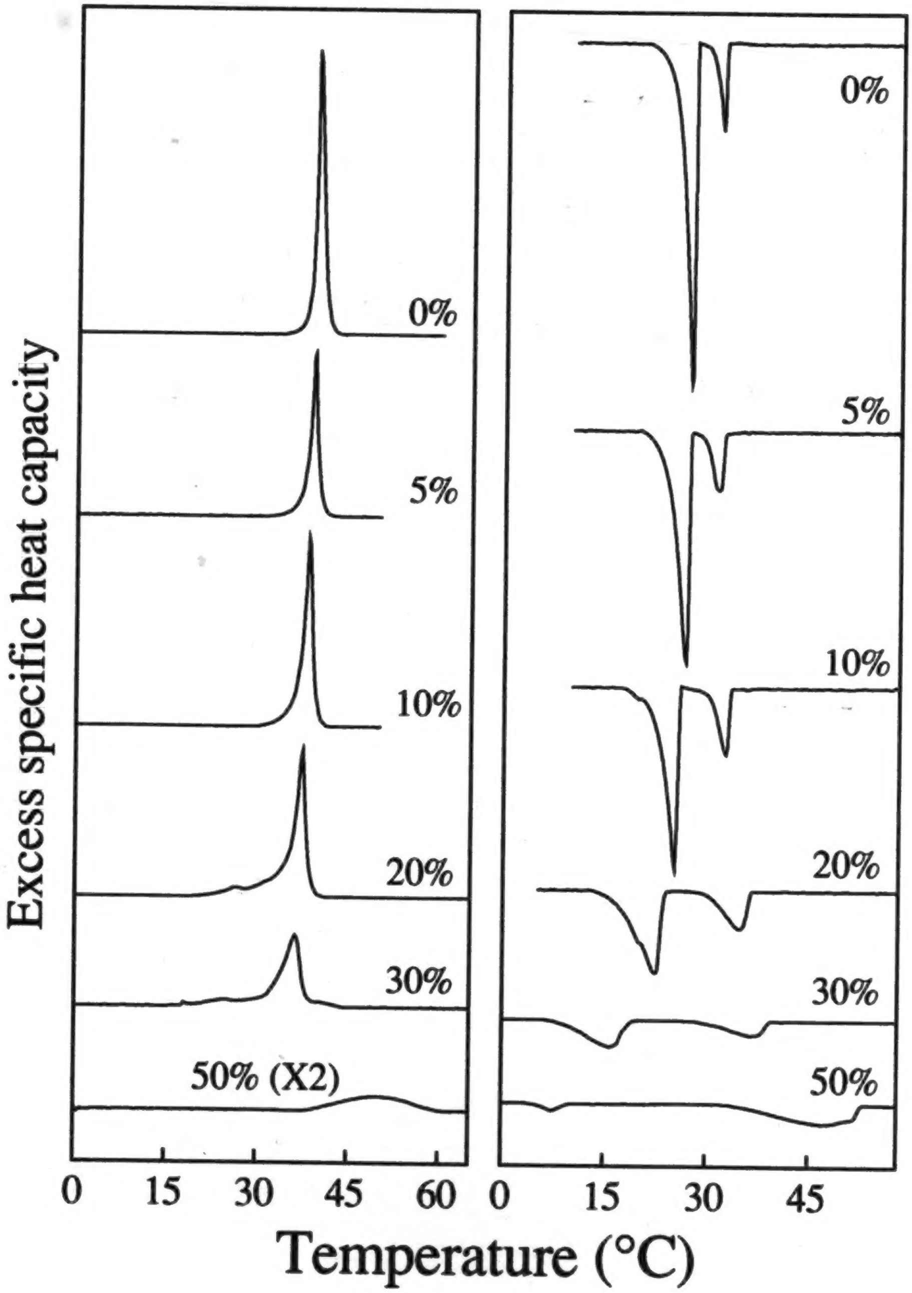
Excess specific heat capacity
0%
5%
10%
20%
30%
50% (X2)
0%
5%
10%
20%
30%
50%
0
15
30
45
60
0
15
30
45
Temperature (°C)

Figure X-8. Representative plots of the temperatures of the L_α/H_{II} (heating ●) and of the H_{II}/L_α (cooling ◊) and L_α/L_β (cooling ○) phase transitions of aqueous dispersions of the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration. Transition temperatures were corrected for differences in heating and cooling scan rates between the various samples analyzed.

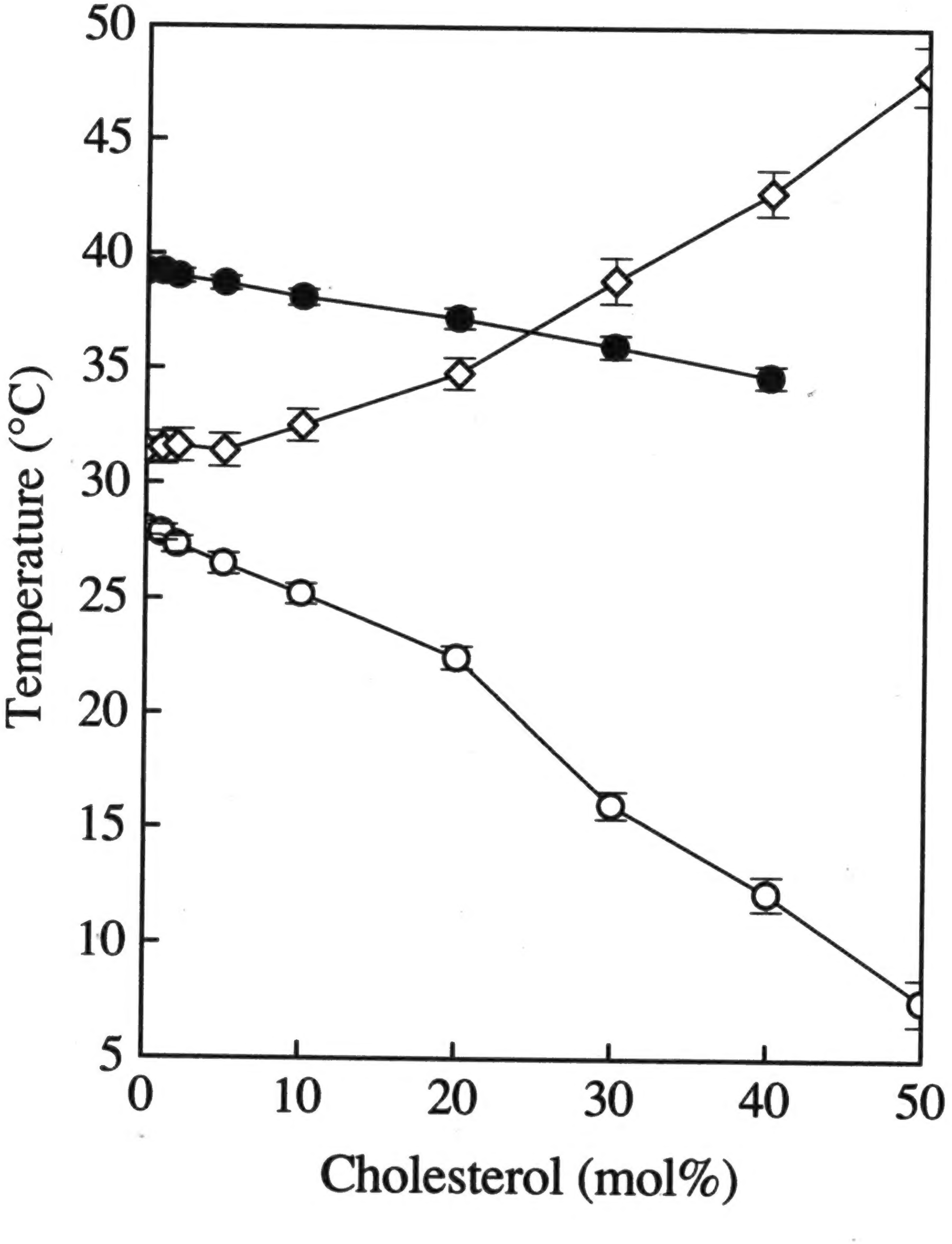
Temperature (°C)
50
45
40
35
30
25
20
15
10
5
0
10
20
30
40
50
Cholesterol (mol%)

Figure X-9. Representative plots of the total enthalpy of the L_c/H_{II} (heating ●) and of the H_{II}/L_α (cooling ◊) and L_α/L_β (cooling ○) phase transitions of aqueous dispersions of the MGDG from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

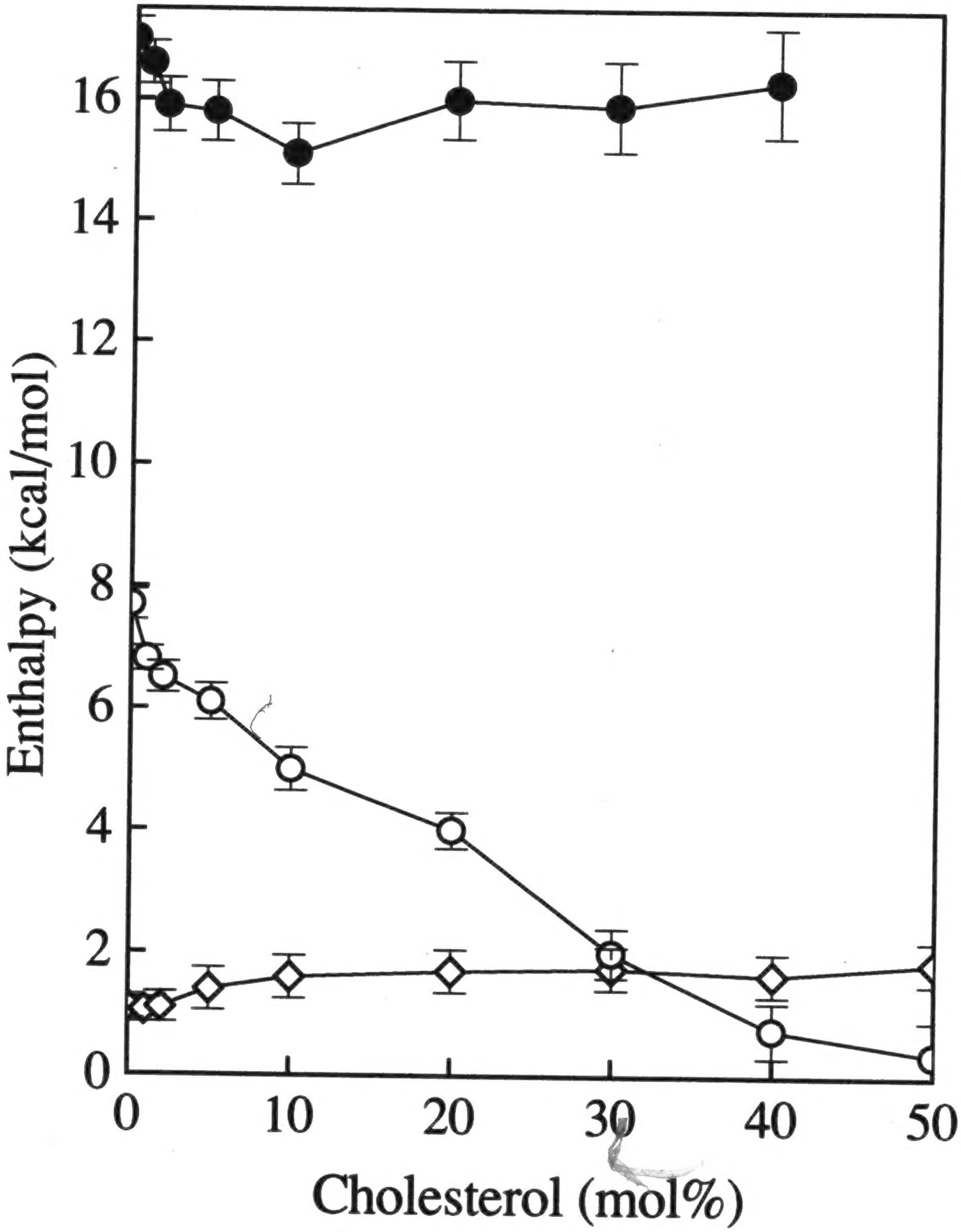
16
14
12
10
8
6
4
2
0
0
10
20
30
40
50
Enthalpy (kcal/mol)
Cholesterol (mol%)

Figure X-10. Representative DSC heating scans of aqueous dispersions of the total polar lipids from elaidic acid-homogeneous *A. laidlawii* membranes containing various concentrations of cholesterol. Cooling scans were essentially identical and are not illustrated.

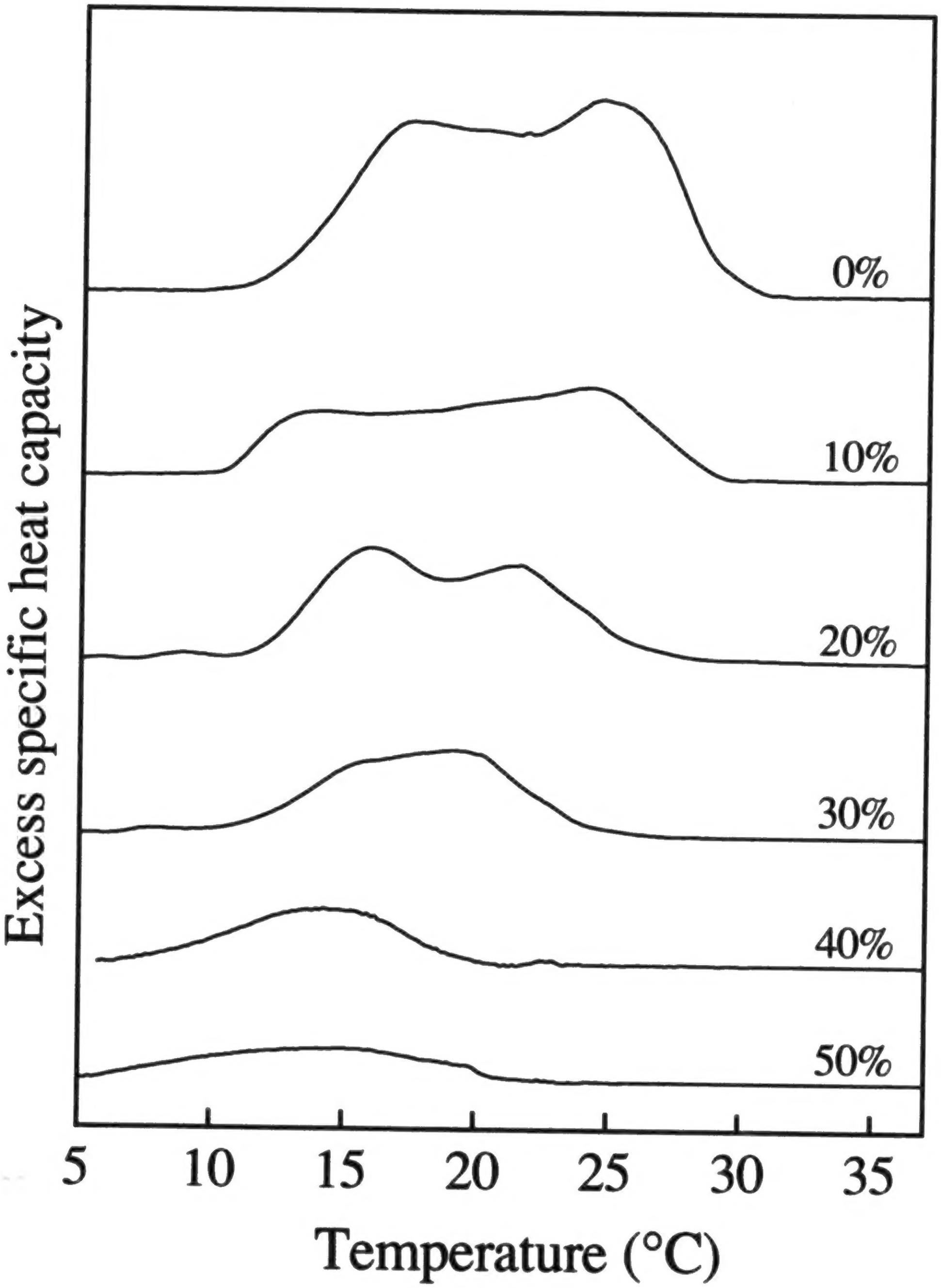
Excess specific heat capacity
0%
10%
20%
30%
40%
50%
5
10
15
20
25
30
35
Temperature (°C)

Figure X-11. Representative plot of the overall chain-melting phase transition midpoint temperature of aqueous dispersions of the total polar lipids from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

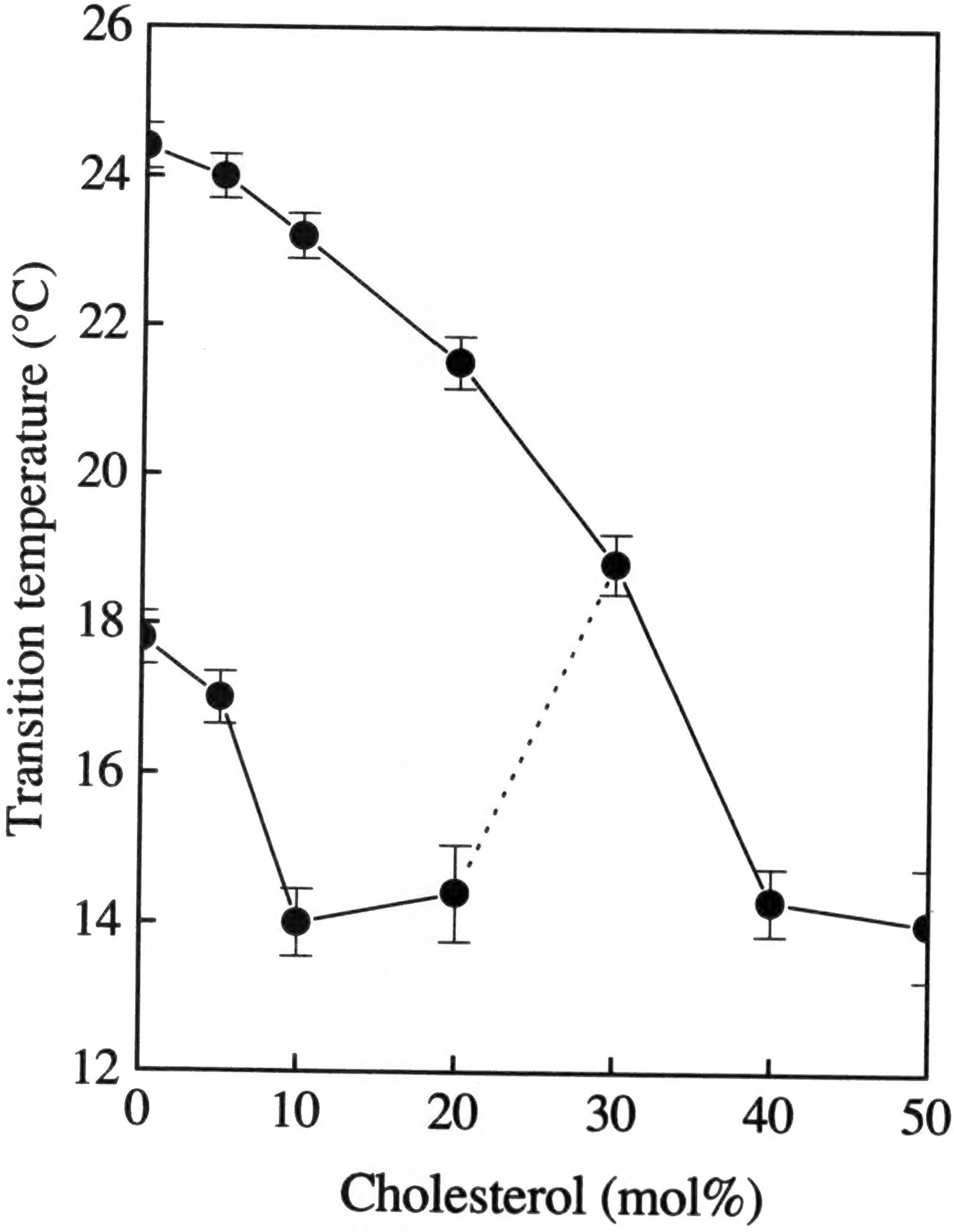
Transition temperature (°C)
26
24
22
20
18
16
14
12
0
10
20
30
40
50
Cholesterol (mol%)

Figure X-12. Representative plot of the total enthalpy of the chain-melting phase transition of aqueous dispersions of the total polar lipids from elaidic acid-homogeneous *A. laidlawii* membranes as a function of cholesterol concentration.

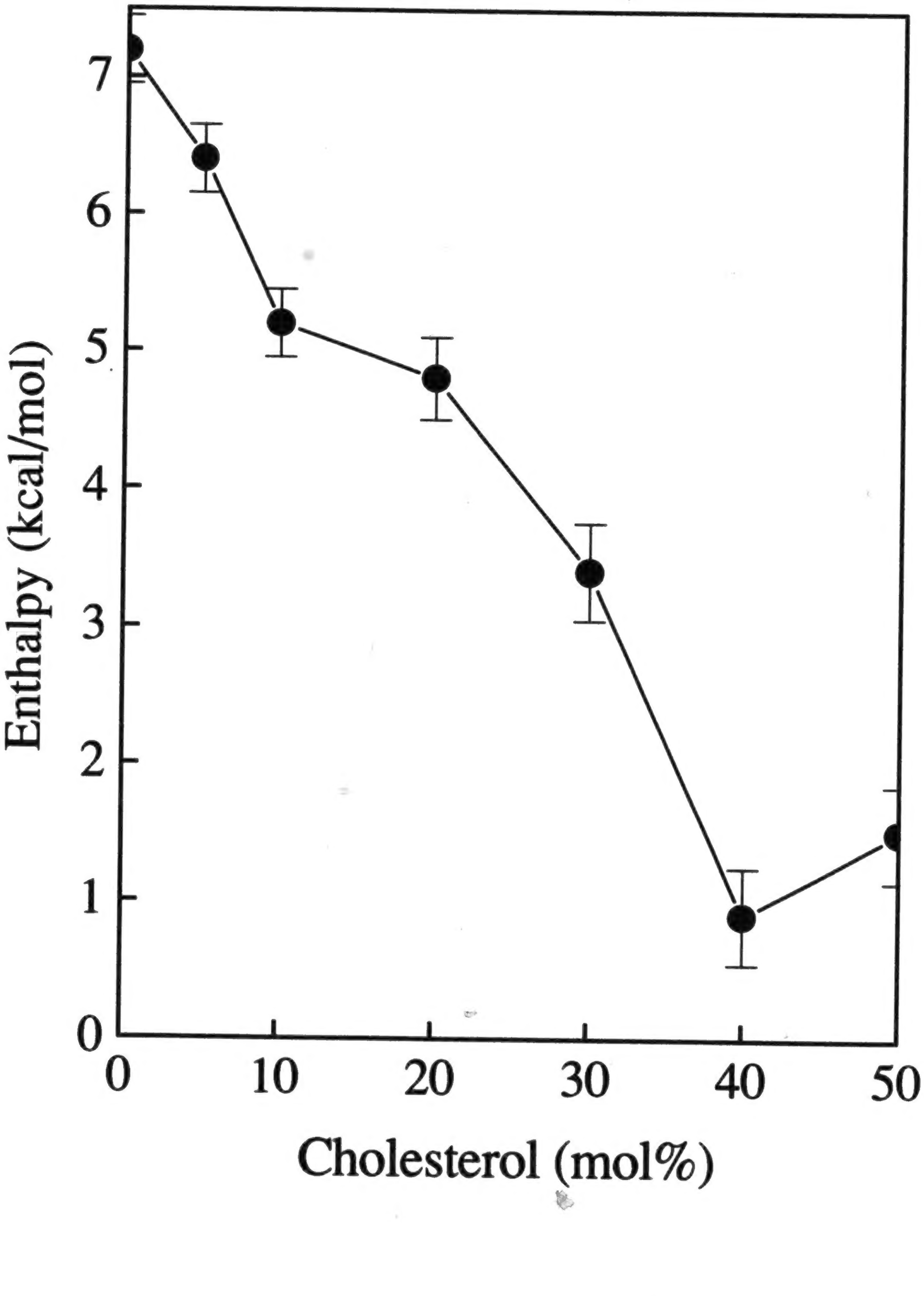
Enthalpy (kcal/mol)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
Cholesterol (mol%)

Table X-1. The polar headgroup composition of the membrane lipids from *A. laidlawii* cells grown in the presence of exogenous elaidic acid and avidin[a]

membrane lipid	quantity present (mol%)	membrane lipid	quantity present (mol%)
APG	1±0.5	PG	27.3±6.0
MGDG	43±6.3	GPMGDG	0.5±0.2
DGDG	20.7±4.7	GPDGDG	7.2±2.0

[a] Values presented are the arithmetic means (averages) and standard deviations from the mean of three independent experiments.

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CHAPTER XI. GENERAL DISCUSSION AND CONCLUSIONS

Membranes play a central role in the structure and function of all cells, and in turn, cholesterol plays a central role in the structure and function of animal cell membranes. It is widely acknowledged that the major role of cholesterol in biological membranes is as a passive modulator of the physical properties of the host bilayer membrane (Nes and McKean, 1977; Yeagle, 1985, 1988; Vist and Davis, 1990). Prior knowledge of the unique physical properties and phase state of cholesterol-rich phospholipid domains is based primarily on studies of cholesterol/DMPC and cholesterol/DPPC model membrane mixtures (Vist and Davis, 1990). Known as the liquid-ordered or β-phase, cholesterol-rich DMPC or DPPC domains exhibit temperature-independent physical properties and a homogeneous lateral distribution of cholesterol (Engleman and Rothman, 1973; Chong, 1994; Tang *et al.*, 1994). Moreover, the nature of the β-phase is thought to be relatively independent of the length or chemical structure of the fatty acyl chains of the host PC bilayer or the structure of the lipid polar headgroups (Ipsen *et al.*, 1987; Gennis, 1989; Thewalt and Bloom, 1992; Corvera *et al.*, 1992; Linseisen *et al.*, 1993). This liquid-ordered or β-phase was postulated to be the relevant physical state for the complex mixtures of phospholipids found in biological membranes containing appreciable levels of cholesterol (Ipsen *et al.*, 1987; Thewalt and Bloom, 1992; Linseisen *et al.*, 1993). However, I have shown that the phase behavior and organization of cholesterol/phospholipid mixtures depends on the structure of the phospholipid polar headgroup and on the structure and length of the hydrocarbon chains. Consequently, studies of the β-phase of PC bilayers, regardless of their breadth or number, will not accurately model the physical properties of biological membranes containing appreciable levels of cholesterol. Furthermore, I have shown that differences in cholesterol-phospholipid and cholesterol-glycolipid interactions may also lead to variations in the lateral distribution of cholesterol within the host membrane. Thus I suggest that the existence of cholesterol-rich and cholesterol-poor domains in biological membranes may be a consequence of the differences of the affinity of cholesterol for different phospholipids or glycolipids. Moreover, variations in the size and composition of cholesterol-rich and cholesterol-poor domains may directly or indirectly regulate the function of membrane proteins and the transport and metabolism of cholesterol within eukaryotic cells.

Cholesterol-phospholipid interactions in model membrane systems

With respect to PC hydrocarbon chain length, I found that the stoichiometry of cholesterol-PC interactions does not vary significantly with physiologically relevant changes in the length of fully saturated hydrocarbon chains of the host PC bilayer (McMullen *et al.*, 1993), in contrast to prior reports which erroneously reported that the stoichiometry of cholesterol-PC interactions decreases with PC chain length (Singer and Finegold, 1990). However, variations in the mismatch between the length of the sterol molecule and the hydrophobic thickness of the host PC bilayer will markedly alter the temperature and cooperativity of the chain-melting transition of cholesterol-rich PC domains. The chain length-dependent phase behavior of cholesterol/PC mixtures lead to our newly proposed cholesterol/PC phase diagram in which, contrary to prior cholesterol/PC phase diagrams, there is no eutectic point is observed near 7.5 mol% cholesterol and the phase boundaries of cholesterol-rich PC domains exhibit a marked dependence on both temperature and PC hydrocarbon chain length (McMullen and McElhaney, 1995). Temperature-induced changes in the structure of the β-phase include increased hydrocarbon chain disorder, decreased bilayer thickness, and possibly a shift in the location of the cholesterol molecule towards the bilayer interface (Reinl *et al.*, 1992; Chia *et al.*, 1993; Huang *et al.*, 1993). In more extreme cases of cholesterol/PC or sterol/PC hydrophobic mismatch, I found that the miscibility of the sterol, and consequently the magnitude of the effect of the sterol on the physical properties of the host bilayer, decreases markedly (McMullen *et al.*, 1994, 1995; Vilchèze *et al.*, 1996). In fact with androstenol, a sterol lacking the C17 alkyl side chain, I found that even small degrees of androstenol-PC hydrophobic mismatch lead to extensive lateral phase separation of the sterol in both gel and liquid-crystalline state PC bilayers. Thus I demonstrated for the first time that sterol/PC hydrophobic mismatch influences not only the effect of the sterol on the physical properties of the host bilayer, but also the miscibility of the sterol within the host bilayer and thus the effective stoichiometry of cholesterol-phospholipid interactions (see also Mattjus *et al.*, 1994, 1995; Slotte *et al.*, 1994). This suggests that an important function of the alkyl side chain of cholesterol is to adjust to variations in the thickness of the host bilayer so as to prevent large scale lateral phase separation of cholesterol in biological membranes.

I have also shown that the effect of cholesterol on the phase behavior and organization of the host bilayer varies markedly with the structure of the phospholipid headgroup. In particular, I found that for a given hydrocarbon chain length, the miscibility of cholesterol in the host phospholipid bilayer is inversely proportional to the extent of inter-headgroup hydrogen bonding and electrostatic interactions in phospholipid bilayers, and thus the maximum amount of cholesterol which can be dispersed in phospholipid bilayer model membranes decreases in the order PC>PS>PE (McMullen *et al.*, 1997a,b,c). Furthermore, cholesterol also appears to facilitate the formation of highly ordered, cholesterol-poor crystalline phospholipid arrays in PE and PS bilayers. Therefore, in contrast to cholesterol/PC mixtures, the effective stoichiometry of cholesterol-PS and cholesterol-PE interactions decreases with decreasing temperature and increasing cholesterol levels. Even at cholesterol concentrations approaching 50 mol%, cholesterol/PE and cholesterol/PS mixtures consist of a mosaic of cholesterol-rich and cholesterol-poor phospholipid domains whose relative proportions vary markedly with the phase state and temperature of the host lipid bilayer. However, it is important to note that when a single *trans*-double bond is introduced into the hydrocarbon chains of the host phospholipid bilayer, subsequently increasing the spacing of and decreasing the attractive interactions between neighboring phospholipid headgroups, the miscibility of cholesterol increases markedly relative to the corresponding saturated phospholipid (McMullen *et al.*, 1997c).

Finally, I have proposed a comprehensive model for cholesterol-phospholipid interactions based on my studies of the effect of cholesterol on the thermotropic phase behavior and organization of a variety of lipid bilayer systems. In this model cholesterol-phospholipid interactions are favored when the hydrophobic lengths of the cholesterol and the phospholipid molecules are closely matched and when there is minimal hydrogen bonding and electrostatic attractive interactions between phospholipid molecules.

Conversely, when the attractive interactions between the headgroups of phospholipid molecules are extensive and/or the cholesterol-phospholipid hydrophobic mismatch is extreme, cholesterol-phospholipid interactions are progressively less favored. Thus in mono-unsaturated or saturated PC bilayers of moderate hydrocarbon chain lengths (i.e. 17 carbons), cholesterol-phospholipid interactions are favored over phospholipid-phospholipid interactions and the miscibility of cholesterol in the host bilayer approaches that of an ideal system as illustrated at the top of Figure XI-1. Under these circumstances the effect of cholesterol on the physical properties of the membrane are the most pronounced. Conversely, in PE or PS bilayers with progressively longer hydrocarbon chains, phospholipid-phospholipid interactions are heavily favored over cholesterol-phospholipid interactions and as a result there is extensive lateral phase separation of cholesterol in the host phospholipid bilayer as illustrated at the bottom of Figure XI-1. The lateral phase separation of cholesterol in PE and PS bilayers reduces the number of effective cholesterol/PE or cholesterol/PS contacts. Consequently, the effect of cholesterol on the physical properties of the host membrane is markedly attenuated relative to that observed in corresponding cholesterol/PC mixtures. The lateral distribution of cholesterol, and in turn the magnitude of the effect of cholesterol on the physical properties of the host phospholipid bilayer, will progressively vary between these two extreme situations depending on the composition, temperature and phase state of the phospholipid bilayer. Interestingly, the thermotropic phase behavior and organization of PC, PE and PS bilayers containing α-helical, hydrophobic transmembrane peptides is qualitatively similar to that observed for cholesterol/PC, cholesterol/PE and cholesterol/PS bilayers (Zhang *et al.*, 1992, 1995a,b, Nezil and Bloom, 1992). Thus degree of hydrophobic mismatch and the strength of phospholipid inter-headgroup interactions may be particularly important in defining the nature and magnitude of the interactions between phospholipids, cholesterol and proteins in biological membranes. I have in fact demonstrated that the existence of two distinct pools of cholesterol in the membranes of *A. laidlawii* cells (Monck *et al.*, 1993) may be a direct consequence of the poor miscibility of cholesterol in bilayers composed primarily of glycolipids which, in turn, leads to the lateral phase separation of cholesterol within the bilayer (McMullen *et al.*, 1997d). This is fully consistent with the model developed for cholesterol-phospholipid interactions based on our model systems and affirms its extension to a biological membrane.

From an experimental perspective, it is important to note that subsequent studies of cholesterol/phospholipid mixtures should not assume that the miscibility of cholesterol, and the effect of cholesterol on the physical state of the host lipid bilayer, is independent of the composition of the host membrane, or its temperature or its cholesterol content. It is clear that increasing levels of cholesterol may progressively decrease or increase the phase transition temperature, thus varying the phase state of the host phospholipid bilayer, which in turn may affect the results of biophysical measurements made at constant temperature. Furthermore, the magnitude of the effect of cholesterol on the physical properties of different host phospholipid bilayers may also vary with temperature and phase state due to changes in the lateral distribution of cholesterol within the membrane. I (McMullen and McElhaney, 1996; McMullen *et al.*, 1997a,b), and others (Ali *et al.*, 1994; Smaby *et al.*, 1994), have identified numerous examples of biophysical studies of cholesterol/phospholipid binary mixtures in which the conclusions are partially or wholly invalidated by the fact that the investigators were not aware of changes in the phase state of the cholesterol/phospholipid mixture or in the effective stoichiometry of cholesterol-phospholipid interactions. Since this work delineates how the miscibility of cholesterol varies as function of the composition, phase state and temperature of different lipid bilayers, it should serve as an important aid in the proper interpretation of subsequent biophysical studies of the physical properties and organization of cholesterol/phospholipid or cholesterol/glycolipid mixtures.

Cholesterol-phospholipid interactions in biological membranes

A number of different models for cholesterol-phospholipid interactions in biological membranes have been proposed since the studies of cholesterol-phospholipid interactions first emerged (see Hui, 1988; Tang *et al.*, 1994; Chong, 1995; McMullen and McElhaney, 1995; 1996). Generally these models, based almost exclusively on studies of cholesterol/PC model membrane systems, suggest that cholesterol is fully miscible in biological membranes up to 50 mol% cholesterol and that the lateral distribution of cholesterol in the membrane is homogeneous. As a result, cholesterol is predicted to have a uniform effect on the fluidity, phase state and permeability of biological membranes, regardless of the composition of the membrane (Corvera *et al.*, 1992; Thewalt and Bloom, 1992; Linseisen *et al.*, 1993). However, I have clearly demonstrated that both the miscibility and the lateral distribution of cholesterol in both model and *Acholeplasma laidlawii* B membranes will vary with changes in the length of the phospholipid hydrocarbon chains or with variations in the membrane polar headgroup composition. Thus the ability of cholesterol to modulate the permeability, phase state and fluidity of a biological membrane may depend, in part, on the lipid composition of the host membrane (McMullen *et al.*, 1995). Moreover, the differential affinity of cholesterol for different phospholipids may also drive the formation of localized cholesterol-rich and cholesterol-poor phospholipid domains within a given membrane. Phase separation in biological membranes is considered to be an important mechanism by which the cell can modulate membrane function (Glaser, 1993; Vaz and Alameida, 1993; Kinnunen *et al.*, 1994; Welti and Glaser, 1994). Distinct cholesterol-rich or cholesterol-poor phospholipid domains may modulate localized phenomena such as membrane budding and fission or regulate bimolecular reactions within the bilayer (Yeagle, 1988; Glaser, 1993; Vaz and Alameida, 1993; Kinnunen *et al.*, 1994; Welti and Glaser, 1994). With respect to membrane budding/fission, experimental studies have shown that cholesterol/phospholipid mixtures exhibiting various degrees of lateral phase separation (cholesterol-rich and cholesterol-poor domains) may undergo dramatic shape fluctuations, which can lead to vesicular fission without the assistance of membrane-associated proteins (Julicher and Lipowsky, 1993; Dobereiner *et al.*, 1993). Thus variations in the size and composition of the cholesterol-rich and cholesterol-poor domains within the intracellular membranes of different organelles may modulate the rate of budding and the size of the vesicles (Julicher and Lipowsky, 1993; Dobereiner *et al.*, 1993). The phase separation of cholesterol-rich and cholesterol-poor phospholipid domains may also control the location and rate of bimolecular reactions within the bilayer (Thompson *et al.*, 1992; Thompson, 1993; Vaz and Alameida, 1993; Welti and Glaser, 1994). Differences in the physical properties and phase state of cholesterol-rich and cholesterol-poor domains in biological membranes could provide an excellent means of sorting reactants or proteins and their substrates. Bimolecular reactions could be facilitated by co-localizing the reactants or a membrane protein and its substrate within the same domain, or inhibited by isolating the reactants in different domains (Vaz and Alameida, 1993). Overall, cholesterol is particularly well suited for regulating bimolecular reactions or membrane budding/fission events given the ability of cholesterol to form laterally phase separated $L_{o\alpha}/L_{\alpha}$ (cholesterol/PC) or cholesterol-rich and cholesterol-poor domains (cholesterol/PS and cholesterol/PE mixtures), even in liquid-crystalline state (McMullen and McElhaney, 1996; Julicher and Lipowsky, 1993). Thus the heterogeneous distribution of cholesterol in biomembranes may allow for local variations in the physical properties of the bilayer to accommodate activities such as bilayer fusion/fission or phospholipase activity, while also maintaining the proper fluidity and phase state of the membrane as a whole (Gennis, 1989; Julicher and Lipowsky, 1993; Vaz and Alameida, 1993; Welti and Glaser, 1994; Burack and Biltonen, 1994).

In addition, variations in the miscibility of cholesterol in different host phospholipid bilayers may also have a significant impact on the distribution of cholesterol between different intracellular membranes of within eukaryotic cells. My results suggest that the steady-state distribution of cholesterol may be governed, in part, by the relative affinity of cholesterol for the phospholipid components of different intracellular membranes (Schroeder *et al.*, 1991, 1995; Liscum, 1992; van Meer, 1993; Liscum and Underwood, 1995; Lange and Steck, 1996; Yeagle, 1988). Thus the asymmetric distribution of cholesterol between the plasma and intracellular membranes of eukaryotic cells (see chapter I) may be a consequence of the phospholipid composition of the various cell membranes (Yeagle and Young, 1986; van Meer, 1993). For example membranes containing high levels of SPM or PC, such as the plasma membrane of eukaryotic cells, typically contain much higher levels of cholesterol than the intracellular membranes which contain relatively higher levels of PE and PS (White, 1973). The relationship between cholesterol and phospholipid levels in the membranes of eukaryotic cells is consistent with the results of our study where the affinity of cholesterol for different phospholipid species decreases in the order PC>>PE=PS. This model for the steady-state distribution of cholesterol between the plasma and intracellular membranes is particularly attractive because the system is inherently self-organizing. However, it is important to note that it is only one part of the cell machinery responsible for the sorting and transport of cholesterol within eukaryotic cells. Other components of this system are considered in the following section.

Cholesterol-protein interactions in biological membranes

Cholesterol has also been shown to modulate the activity of membrane proteins. Cholesterol may regulate the activity of membrane proteins directly via specific cholesterol-protein interactions (Whetton *et al.*, 1983; Yeagle, 1988; Narayanaswami *et al.*, 1993; Fernandez-Ballester *et al.*, 1994; Ding *et al.*, 1994; Liang *et al.*, 1995; Gimpl *et al.*, 1995; Klein *et al.*, 1995; Murata *et al.*, 1995; Arnold and Cornell, 1996) or indirectly via changes in the physical properties of the host membrane (Muhlebach and Cherry, 1982; Nunez and Glass, 1982; Ortega and Mas-Oliva, 1984; Vermuri and Philipson, 1989; Mitchell *et al.*, 1990; Yeagle, 1991; George and McElhaney, 1992; Mitchell *et al.*, 1992). With respect to the bulk effects of cholesterol on the host membrane, cholesterol may modulate the activity of membrane proteins indirectly via variations in the hydrocarbon chain packing, fluidity, surface charge density, interfacial hydration, and local curvature of the membrane (Yeagle, 1988; Mitchell *et al.*, 1990; Yeagle 1991; George and McElhaney, 1992; Mitchell *et al.*, 1992). There is a clear correlation between the level of cholesterol, the fluidity and phase state of biological membranes and the activity of membrane proteins (Dahl and Dahl, 1988; Yeagle, 1988; Mitchell *et al.*, 1990; George and McElhaney, 1992). However, because of the complex nature of the effects of cholesterol on the physical properties of the membrane, it has been difficult to determine how changes in the phase state and organization of the bilayer are propagated to membrane proteins. Until now, it was hypothesized that cholesterol indirectly modulates the activity of membrane proteins either by altering the conformation of membrane proteins via bulk changes in the orientational order and packing of the phospholipids of the membrane (Rooney *et al.*, 1984; Yeagle, 1988; George and McElhaney, 1992; Ortega *et al.*, 1996), or by reducing the free volume available for protein conformational changes (Mitchell *et al.*, 1990;

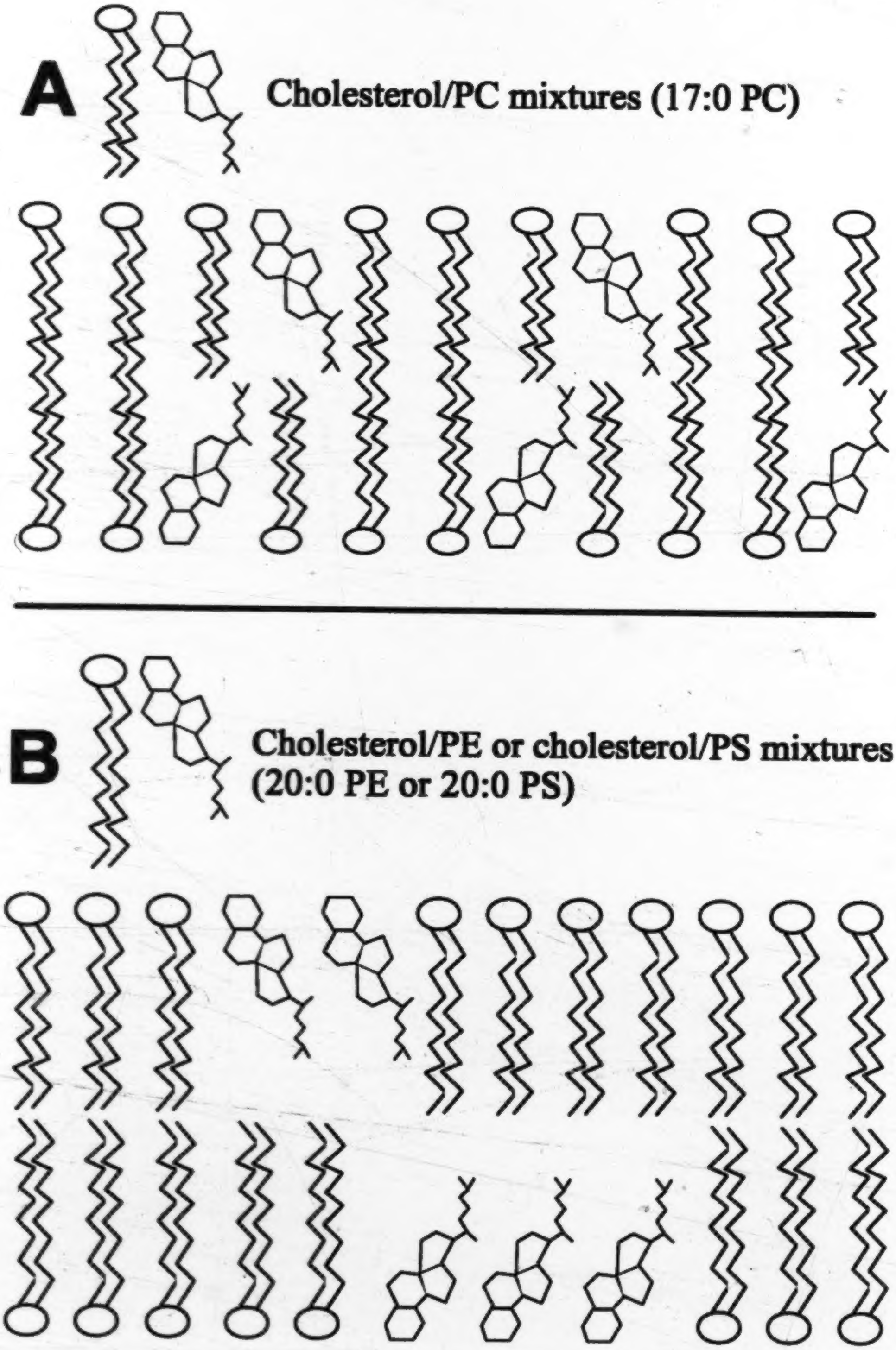
Yeagle, 1991). The results of my work, coupled with the recent findings that certain membrane proteins may interact preferentially with cholesterol-rich phospholipid domains (Bretscher and Munro 1993; Fielding and Fielding 1996; Hua *et al.*, 1996; Murata *et al.*, 1996; Smart *et al.*, 1996), suggest another possible mechanism for modulation of protein activity. I have shown that cholesterol is not evenly distributed within phospholipid membranes and that cholesterol-rich and cholesterol-poor phospholipid domains may exist up to 50 mol% cholesterol, depending on the composition of the membrane. It has also been shown that the activity of the membrane proteins HMG-CoA reductase and SCAP, both of which are central to the regulation of cholesterol metabolism in animal cells, may be modulated by the presence of cholesterol (Davis and Poznansky, 1987; Goldstein and Brown, 1990; Liscum and Underwood, 1995; Lange and Steck, 1994, 1996; Hua *et al.*, 1996). Thus, I suggest that changes in the size and composition of cholesterol-rich and cholesterol-poor domains in the intracellular membranes of eukaryotic cells may modulate the activity of the membrane proteins HMG-CoA reductase and SCAP which, in turn, regulate the production of cholesterol (Liscum and Underwood, 1995). This is consistent with the work of Lange and Steck (1996) which indicates that the mechanism for sensing cholesterol levels in eukaryotic cells and mediating changes in the cellular metabolism of cholesterol are located within the plasma and/or the endoplasmic reticulum membranes (Lange and Steck, 1996). Since there are an increasing number of membrane proteins whose function may be mediated by cholesterol (Narayanaswami *et al.*, 1993; Fernandez-Ballester *et al.*, 1994; Ding *et al.*, 1994; Liang *et al.*, 1995; Gimpl *et al.*, 1995; Klein *et al.*, 1995; Murata *et al.*, 1995), variations in the size and composition of the cholesterol-rich and cholesterol-poor phospholipid domains in eukaryotic cell membranes may be an important mechanism by which the activity of membrane proteins, as well as the processes of cell metabolism and growth, are regulated.

The lateral segregation of cholesterol within phospholipid bilayers may also form the basis of a selective sorting system for the transport of membrane proteins and cholesterol, via vesicular transport, from the endoplasmic reticulum to the plasma membrane. For example, the segregation of cholesterol-rich and cholesterol-poor phospholipid domains may serve as a sorting mechanism for integral and GPI-linked membrane proteins destined for the plasma membrane (Rothberg *et al.*, 1990; Bretscher and Munro 1993; Rogers and Glaser, 1993; van Meer, 1993). This process is postulated to occur as a consequence of the greater hydrophobic thickness and/or unique physical properties of the more ordered cholesterol-containing phospholipid domains which selects for membrane proteins with longer transmembrane segments. In addition, the membrane protein caveolin, known to preferentially localize to regions of intracellular membranes rich in cholesterol and PC, has been linked to the rapid transport of cholesterol from the endoplasmic reticulum to the plasma membrane (Fielding and Fielding, 1995; Murata *et al.*, 1996; Smart et al., 1996). Furthermore, distinct cholesterol-rich and cholesterol-poor phospholipid domains in the plasma membrane of eukaryotic cells may mediate the association of apolipoproteins to the plasma membrane, thus modulating the efflux of cholesterol from the cell via HDL (Rothblat *et al.*, 1992; Davidson *et al.*, 1995). These studies, coupled with my work, suggest that membrane proteins can utilize cholesterol-rich domains in biological membranes to selectively transport proteins or cholesterol between membranes or to regulate the activity of membrane proteins (Liscum and Underwood, 1995; Lange and Steck, 1996; Fielding and Fielding, 1995; Murata *et al.*, 1996). For these mechanisms to be effective, the lateral phase separation of cholesterol-rich and cholesterol-poor domains would have to occur at low cholesterol concentrations to allow for selective concentration of cholesterol and proteins from the endoplasmic reticulum through the Golgi network towards the plasma membrane. My work clearly demonstrates that cholesterol-induced phase separation in cholesterol/phospholipid mixtures is possible even at very low cholesterol concentrations, in contrast to previous findings (Vist and Davis, 1990). In summary, I suggest that the phase behavior and organization of cholesterol-containing membranes may have an important role to play in the movement of cholesterol and membrane proteins within eukaryotic cells and in the regulation of the activity of the membrane proteins essential for cholesterol homeostasis.

The direction of future investigations

By establishing the unique interactions of cholesterol with individual phospholipids, this work provides a reference point for examining more complex cholesterol/phospholipid or cholesterol/glycolipid mixtures characteristic of biological membranes. In particular, having clarified the nature of cholesterol-phospholipid interactions in binary systems, the next step is to examine how the distribution of cholesterol varies with the mol fraction of zwitterionic and anionic lipids in gel and liquid-crystalline state ternary cholesterol/phospholipid mixtures using both HS-DSC and FTIR. For the FTIR experiments, the use of isotopically labeled phospholipids would allow the investigator to follow the separate effects of cholesterol on each phospholipid in the mixture, and should provide a wealth of information pertaining directly to the thermotropic phase behavior and organization of multicomponent cholesterol/phospholipid mixtures. Furthermore, the use of magic angle spinning-NMR, a newly emerging technique, on a much wider range of different cholesterol/phospholipid mixtures is urgently required, as thus far only cholesterol/DPPC systems have been examined (Guerneve *et al.*, 1994; Guo and Hamilton, 1995). This technique, as a complement to the HS-DSC and FTIR analysis, enables the investigator to examine the effect of cholesterol on the orientational order and mobility of various portions of the phospholipid molecule in both cholesterol-rich or cholesterol-poor phospholipid domains. Moreover this technique could confirm the existence of the laterally phase separated domains of cholesterol, which have been indirectly shown by this work, in cholesterol/PE and cholesterol/PS mixtures with varying phospholipid hydrocarbon chain lengths. Similarly, experiments using epifluorescence or time-resolved fluorescent probes could also directly determine how the morphology and size of cholesterol-rich and cholesterol-poor domains vary in different cholesterol/phospholipid mixtures, as indirectly indicated by this volume of work (Chong, 1994; Mattjus *et al.*, 1994; Slotte *et al.*, 1994; Tang *et al.*, 1995; Mattjus *et al.*, 1995; Slotte, 1995). I suggest that future investigations in this area utilize a systematic and multitechnique approach to the study of the complex nature of cholesterol-phospholipid interactions. This approach will fully address the nature and magnitude of the physicochemical forces which govern cholesterol-phospholipid interactions and thus how cholesterol-phospholipid interactions contribute to the distribution and function of cholesterol within eukaryotic cell membranes.

Figure XI-1. Representation of the lateral organization of cholesterol in different phospholipid bilayers. Under conditions where cholesterol-phospholipid interactions are favored, such as in cholesterol/17:0 PC mixtures, the lateral distribution of cholesterol is homogeneous within the bilayer as shown in **A**. Conversely, under conditions where phospholipid-phospholipid interactions are favored, such as in cholesterol/20:0 PE or cholesterol/20:0 PS mixtures, cholesterol exhibits lateral phase separation within the bilayer as shown in **B**. In biological membranes the actual distribution of cholesterol will vary between these two extremes depending on phospholipid composition of the membrane.


A
Cholesterol/PC mixtures (17:0 PC)
B
Cholesterol/PE or cholesterol/PS mixtures
(20:0 PE or 20:0 PS)

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